population of conjugates of the invention are attached to multiple copies of a structurally

identical amino acid or glycosyl residue. Thus, in a second aspect, the invention provides a

peptide conjugate having a population of water-soluble polymer moieties, which are

covalently linked to the peptide through an intact glycosyl linking group. In a preferred

5   conjugate of the invention, essentially each member of the population is linked via the

glycosyl linking group to a glycosyl residue of the peptide, and each glycosyl residue of the

peptide to which the glycosyl linking group is attached has the same structure.

        Also provided is a peptide conjugate having a population of water-soluble polymer

moieties covalently linked thereto through an intact glycosyl linking group. In a preferred

10   embodiment, essentially every member of the population of water soluble polymer moieties

is linked to an amino acid residue of the peptide via an intact glycosyl linking group, and

each amino acid residue having an intact glycosyl linking group attached thereto has the same

structure.

        The present invention also provides conjugates analogous to those described above in

15   which the peptide is conjugated to a therapeutic moiety, diagnostic moiety, targeting moiety,

toxin moiety or the like via an intact glycosyl linking group. Each of the above-recited

moieties can be a small molecule, natural polymer (e.g., peptide) or synthetic polymer.

        In an exemplary embodiment, interleukin-2 (IL-2) is conjugated to transferrin via a

bifunctional linker that includes an intact glycosyl linking group at each terminus of the PEG

20   moiety (Scheme 1). For example, one terminus of the PEG linker is functionalized with an

intact sialic acid linker that is attached to transferrin and the other is functionalized with an

intact GalNAc linker that is attached to IL-2.

        In another exemplary embodiment, EPO is conjugated to transferrin. In another

exemplary embodiment, EPO is conjugated to glial derived neurotropic growth factor

25   (GDNF). In these embodiments, each conjugation is accomplished via a bifunctional linker

that includes an intact glycosyl linking group at each terminus of the PEG moiety, as

aforementioned. Transferrin transfers the protein across the blood brain barrier.

        As set forth in the Figures appended hereto, the conjugates of the invention can

include intact glycosyl linking groups that are mono- or multi-valent (e.g., antennary

30   structures), see, Figures 14-22. The conjugates of the invention also include glycosyl linking

groups that are O-linked glycans originating from serine or threonine (Figure 11). Thus,

conjugates of the invention include both species in which a selected moiety is attached to a peptide via a monovalent glycosyl linking group. Also included within the invention are conjugates in which more than one selected moiety is attached to a peptide via a multivalent linking group. One or more proteins can be conjugated together to take advantage of their

5   biophysical and biological properties.

In a still further embodiment, the invention provides conjugates that localize selectively in a particular tissue due to the presence of a targeting agent as a component of the conjugate. In an exemplary embodiment, the targeting agent is a protein. Exemplary proteins include transferrin (brain, blood pool), human serum (HS)-glycoprotein (bone, brain,

10   blood pool), antibodies (brain, tissue with antibody-specific antigen, blood pool), coagulation Factors V-XII (damaged tissue, clots, cancer, blood pool), serum proteins, e.g., α-acid glycoprotein, fetuin, α-fetal protein (brain, blood pool), β2-glycoprotein (liver, atherosclerosis plaques, brain, blood pool), G-CSF, GM-CSF, M-CSF, and EPO (immune stimulation, cancers, blood pool, red blood cell overproduction, neuroprotection), and

15   albumin (increase in half-life).

In addition to the conjugates discussed above, the present invention provides methods for preparing these and other conjugates. Thus, in a further aspect, the invention provides a method of forming a covalent conjugate between a selected moiety and a peptide. Additionally, the invention provides methods for targeting conjugates of the invention to a

20   particular tissue or region of the body.

In exemplary embodiments, the conjugate is formed between a water-soluble polymer, a therapeutic moiety, targeting moiety or a biomolecule, and a glycosylated or non-glycosylated peptide. The polymer, therapeutic moiety or biomolecule is conjugated to the peptide via an intact glycosyl linking group, which is interposed between, and covalently

25   linked to both the peptide and the modifying group (e.g., water-soluble polymer). The method includes contacting the peptide with a mixture containing a modified sugar and a glycosyltransferase for which the modified sugar is a substrate. The reaction is conducted under conditions sufficient to form a covalent bond between the modified sugar and the peptide. The sugar moiety of the modified sugar is preferably selected from nucleotide

30   sugars, activated sugars and sugars, which are neither nucleotides nor activated.

In one embodiment, the invention provides a method for linking two or more peptides through a linking group. The linking group is of any useful structure and may be selected from straight-chain and branched chain structures. Preferably, each terminus of the linker, which is attached to a peptide, includes a modified sugar (i.e., a nascent intact glycosyl

5   linking group).

In an exemplary method of the invention, two peptides are linked together via a linker moiety that includes a PEG linker. The construct conforms to the general structure set forth in the cartoon above. As described herein, the construct of the invention includes two intact glycosyl linking groups (i.e., s + t = 1). The focus on a PEG linker that includes two glycosyl

10   groups is for purposes of clarity and should not be interpreted as limiting the identity of linker arms of use in this embodiment of the invention.

Thus, a PEG moiety is functionalized at a first terminus with a first glycosyl unit and at a second terminus with a second glycosyl unit. The first and second glycosyl units are preferably substrates for different transferases, allowing orthogonal attachment of the first

15   and second peptides to the first and second glycosyl units, respectively. In practice, the $(glycosyl)^1$-PEG-$(glycosyl)^2$ linker is contacted with the first peptide and a first transferase for which the first glycosyl unit is a substrate, thereby forming $(peptide)^1$-$(glycosyl)^1$-PEG-$(glycosyl)^2$. The first transferase and/or unreacted peptide is then optionally removed from the reaction mixture. The second peptide and a second transferase

20   for which the second glycosyl unit is a substrate are added to the $(peptide)^1$-$(glycosyl)^1$-PEG-$(glycosyl)^2$ conjugate, forming $(peptide)^1$-$(glycosyl)^1$-PEG-$(glycosyl)^2$-$(peptide)^2$. Those of skill in the art will appreciate that the method outlined above is also applicable to forming conjugates between more than two peptides by, for example, the use of a branched PEG, dendrimer, poly(amino acid),

25   polysaccharide or the like.

As noted previously, in an exemplary embodiment, interleukin-2 (IL-2) is conjugated to transferrin via a bifunctional linker that includes an intact glycosyl linking group at each terminus of the PEG moiety (Scheme 1). The IL-2 conjugate has an *in vivo* half-life that is increased over that of IL-2 alone by virtue of the greater molecular size of the conjugate.

30   Moreover, the conjugation of IL-2 to transferrin serves to selectively target the conjugate to the brain. For example, one terminus of the PEG linker is functionalized with a CMP-sialic

acid and the other is functionalized with an UDP-GalNAc. The linker is combined with IL-2 in the presence of a GalNAc transferase, resulting in the attachment of the GalNAc of the linker arm to a serine and/or threonine residue on the IL-2.

In another exemplary embodiment, transferrin is conjugated to a nucleic acid for use in gene therapy.

Scheme 1
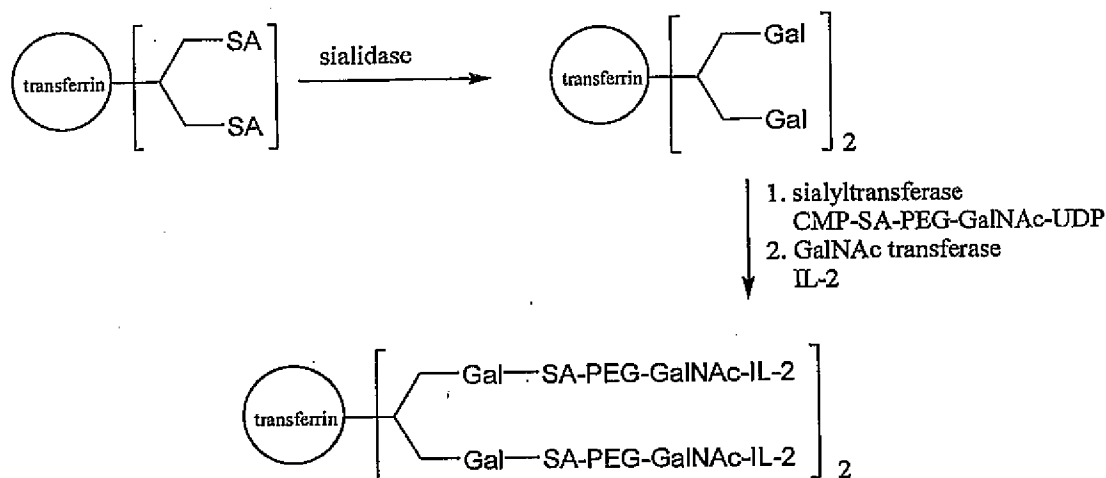
The processes described above can be carried through as many cycles as desired, and is not limited to forming a conjugate between two peptides with a single linker. Moreover, those of skill in the art will appreciate that the reactions functionalizing the intact glycosyl linking groups at the termini of the PEG (or other) linker with the peptide can occur simultaneously in the same reaction vessel, or they can be carried out in a step-wise fashion. When the reactions are carried out in a step-wise manner, the conjugate produced at each step is optionally purified from one or more reaction components (e.g., enzymes, peptides).

A still further exemplary embodiment is set forth in Scheme 2. Scheme 2 shows a method of preparing a conjugate that targets a selected protein, e.g., EPO, to bone and increases the circulatory half-life of the selected protein.
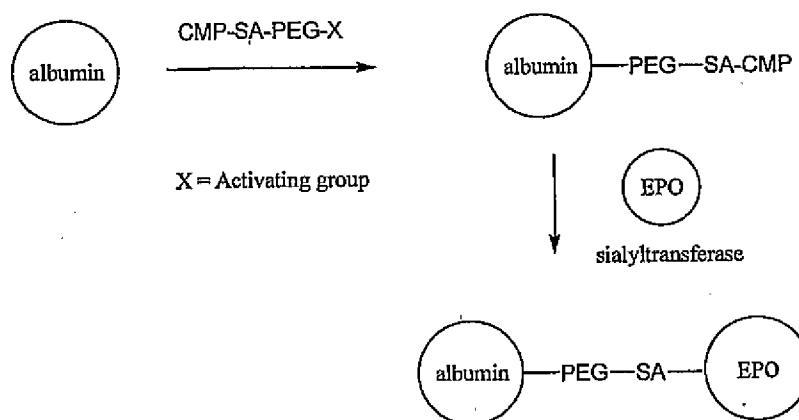
Scheme 2
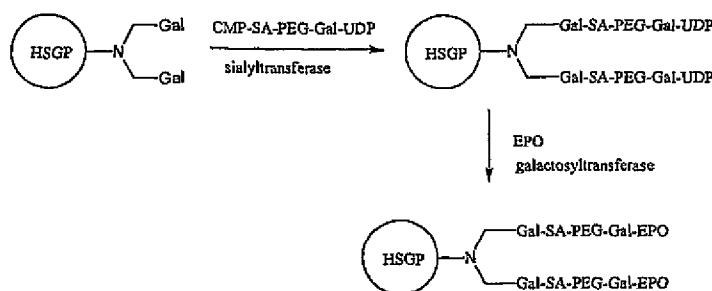
The use of reactive derivatives of PEG (or other linkers) to attach one or more peptide moieties to the linker is within the scope of the present invention. The invention is not limited by the identity of the reactive PEG analogue. Many activated derivatives of poly(ethylene glycol) are available commercially and in the literature. It is well within the abilities of one of skill to choose, and synthesize if necessary, an appropriate activated PEG derivative with which to prepare a substrate useful in the present invention. *See,* Abuchowski *et al. Cancer Biochem. Biophys.,* **7**: 175-186 (1984); Abuchowski *et al., J. Biol. Chem.,* **252**: 3582-3586 (1977); Jackson *et al., Anal. Biochem.,* **165**: 114-127 (1987); Koide *et al., Biochem Biophys. Res. Commun.,* **111**: 659-667 (1983)), tresylate (Nilsson *et al., Methods Enzymol.,* **104**: 56-69 (1984); Delgado *et al., Biotechnol. Appl. Biochem.,* **12**: 119-128 (1990)); N-hydroxysuccinimide derived active esters (Buckmann *et al., Makromol. Chem.,* **182**: 1379-1384 (1981); Joppich *et al., Makromol. Chem.,* **180**: 1381-1384 (1979); Abuchowski *et al., Cancer Biochem. Biophys.,* **7**: 175-186 (1984); Katre *et al. Proc. Natl. Acad. Sci. U.S.A.,* **84**: 1487-1491 (1987); Kitamura *et al., Cancer Res.,* **51**: 4310-4315 (1991); Boccu *et al., Z. Naturforsch.,* **38C**: 94-99 (1983), carbonates (Zalipsky *et al.,* POLY(ETHYLENE GLYCOL) CHEMISTRY: BIOTECHNICAL AND BIOMEDICAL APPLICATIONS, Harris, Ed., Plenum Press, New York, 1992, pp. 347-370; Zalipsky *et al., Biotechnol. Appl. Biochem.,* **15**: 100-114 (1992); Veronese *et al., Appl. Biochem. Biotech.,* **11**: 141-152 (1985)), imidazolyl formates (Beauchamp *et al., Anal. Biochem.,* **131**: 25-33 (1983); Berger *et al., Blood,* **71**: 1641-1647 (1988)), 4-dithiopyridines (Woghiren *et al., Bioconjugate Chem.,* **4**: 314-318 (1993)), isocyanates (Byun *et al., ASAIO Journal,* M649-M-653 (1992)) and epoxides (U.S. Pat. No. 4,806,595, issued to Noishiki *et al.,* (1989). Other linking groups include the urethane linkage between amino groups and activated PEG. *See,* Veronese, *et al., Appl. Biochem. Biotechnol.,* **11**: 141-152 (1985).

In another exemplary embodiment in which a reactive PEG derivative is utilized, the invention provides a method for extending the blood-circulation half-life of a selected peptide, in essence targeting the peptide to the blood pool, by conjugating the peptide to a synthetic or natural polymer of a size sufficient to retard the filtration of the protein by the glomerulus (e.g., albumin). This embodiment of the invention is illustrated in Scheme 3 in which erythropoietin (EPO) is conjugated to albumin via a PEG linker using a combination of chemical and enzymatic modification.

Scheme 3

Thus, as shown in Scheme 3, an amino acid residue of albumin is modified with a reactive PEG derivative, such as X-PEG-(CMP-sialic acid), in which X is an activating group (e.g., active ester, isothiocyanate, etc). The PEG derivative and EPO are combined and contacted with a transferase for which CMP-sialic acid is a substrate. In a further illustrative embodiment, an ε-amine of lysine is reacted with the N-hydroxysuccinimide ester of the PEG-linker to form the albumin conjugate. The CMP-sialic acid of the linker is enzymatically conjugated to an appropriate residue on EPO, e.g., Gal, thereby forming the conjugate. Those of skill will appreciate that the above-described method is not limited to the reaction partners set forth. Moreover, the method can be practiced to form conjugates that include more than two protein moieties by, for example, utilizing a branched linker having more than two termini.

2. Modified Sugars

Modified glycosyl donor species ("modified sugars") are preferably selected from modified sugar nucleotides, activated modified sugars and modified sugars that are simple

saccharides that are neither nucleotides nor activated. Any desired carbohydrate structure can be added to a peptide using the methods of the invention. Typically, the structure will be a monosaccharide, but the present invention is not limited to the use of modified monosaccharide sugars; oligosaccharides and polysaccharides are useful as well.

5   The modifying group is attached to a sugar moiety by enzymatic means, chemical means or a combination thereof, thereby producing a modified sugar. The sugars are substituted at any position that allows for the attachment of the modifying moiety, yet which still allows the sugar to function as a substrate for the enzyme used to ligate the modified sugar to the peptide. In a preferred embodiment, when sialic acid is the sugar, the sialic acid

10   is substituted with the modifying group at either the 9-position on the pyruvyl side chain or at the 5-position on the amine moiety that is normally acetylated in sialic acid.

In certain embodiments of the present invention, a modified sugar nucleotide is utilized to add the modified sugar to the peptide. Exemplary sugar nucleotides that are used in the present invention in their modified form include nucleotide mono-, di- or triphosphates

15   or analogs thereof. In a preferred embodiment, the modified sugar nucleotide is selected from a UDP-glycoside, CMP-glycoside, or a GDP-glycoside. Even more preferably, the modified sugar nucleotide is selected from an UDP-galactose, UDP-galactosamine, UDP-glucose, UDP-glucosamine, GDP-mannose, GDP-fucose, CMP-sialic acid, or CMP-NeuAc. N-acetylamine derivatives of the sugar nucleotides are also of use in the method of the

20   invention.

The invention also provides methods for synthesizing a modified peptide using a modified sugar, e.g., modified-galactose, -fucose, and –sialic acid. When a modified sialic acid is used, either a sialyltransferase or a trans-sialidase (for α2,3-linked sialic acid only) can be used in these methods.

25   In other embodiments, the modified sugar is an activated sugar. Activated modified sugars, which are useful in the present invention are typically glycosides which have been synthetically altered to include an activated leaving group. As used herein, the term "activated leaving group" refers to those moieties, which are easily displaced in enzyme-regulated nucleophilic substitution reactions. Many activated sugars are known in the art.

30   See, for example, Vocadlo et al., In CARBOHYDRATE CHEMISTRY AND BIOLOGY, Vol. 2, Ernst

et al. Ed., Wiley-VCH Verlag: Weinheim, Germany, 2000; Kodama *et al., Tetrahedron Lett.* 34: 6419 (1993); Lougheed, *et al., J. Biol. Chem.* 274: 37717 (1999)).

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Examples of activating groups (leaving groups) include fluoro, chloro, bromo, tosylate ester, mesylate ester, triflate ester and the like. Preferred activated leaving groups, for use in the present invention, are those that do not significantly sterically encumber the enzymatic transfer of the glycoside to the acceptor. Accordingly, preferred embodiments of activated glycoside derivatives include glycosyl fluorides and glycosyl mesylates, with glycosyl fluorides being particularly preferred. Among the glycosyl fluorides, α-galactosyl fluoride, α-mannosyl fluoride, α-glucosyl fluoride, α-fucosyl fluoride, α-xylosyl fluoride, α-sialyl fluoride, α-N-acetylglucosaminyl fluoride, α-N-acetylgalactosaminyl fluoride, β-galactosyl fluoride, β-mannosyl fluoride, β-glucosyl fluoride, β-fucosyl fluoride, β-xylosyl fluoride, β-sialyl fluoride, β-N-acetylglucosaminyl fluoride and β-N-acetylgalactosaminyl fluoride are most preferred.

By way of illustration, glycosyl fluorides can be prepared from the free sugar by first acetylating the sugar and then treating it with HF/pyridine. This generates the thermodynamically most stable anomer of the protected (acetylated) glycosyl fluoride (*i.e.,* the α-glycosyl fluoride). If the less stable anomer (*i.e.,* the β-glycosyl fluoride) is desired, it can be prepared by converting the peracetylated sugar with HBr/HOAc or with HCl to generate the anomeric bromide or chloride. This intermediate is reacted with a fluoride salt such as silver fluoride to generate the glycosyl fluoride. Acetylated glycosyl fluorides may be deprotected by reaction with mild (catalytic) base in methanol (*e.g.* NaOMe/MeOH). In addition, many glycosyl fluorides are commercially available.

Other activated glycosyl derivatives can be prepared using conventional methods known to those of skill in the art. For example, glycosyl mesylates can be prepared by treatment of the fully benzylated hemiacetal form of the sugar with mesyl chloride, followed by catalytic hydrogenation to remove the benzyl groups.

In a further exemplary embodiment, the modified sugar is an oligosaccharide having an antennary structure. In a preferred embodiment, one or more of the termini of the antennae bear the modifying moiety. When more than one modifying moiety is attached to an oligosaccharide having an antennary structure, the oligosaccharide is useful to "amplify"

the modifying moiety; each oligosaccharide unit conjugated to the peptide attaches multiple copies of the modifying group to the peptide. The general structure of a typical chelate of the invention as set forth in the drawing above, encompasses multivalent species resulting from preparing a conjugate of the invention utilizing an antennary structure. Many antennary

5   saccharide structures are known in the art, and the present method can be practiced with them without limitation.

Exemplary modifying groups are discussed below. The modifying groups can be selected for one or more desirable property. Exemplary properties include, but are not limited to, enhanced pharmacokinetics, enhanced pharmacodynamics, improved

10   biodistribution, providing a polyvalent species, improved water solubility, enhanced or diminished lipophilicity, and tissue targeting.

D. Peptide Conjugates

a) Water-Soluble Polymers

The hydrophilicity of a selected peptide is enhanced by conjugation with polar

15   molecules such as amine-, ester-, hydroxyl- and polyhydroxyl-containing molecules. Representative examples include, but are not limited to, polylysine, polyethyleneimine, poly(ethylene glycol) and poly(propyleneglycol). Preferred water-soluble polymers are essentially non-fluorescent, or emit such a minimal amount of fluorescence that they are inappropriate for use as a fluorescent marker in an assay. Polymers that are not naturally

20   occurring sugars may be used. In addition, the use of an otherwise naturally occurring sugar that is modified by covalent attachment of another entity (*e.g.*, poly(ethylene glycol), poly(propylene glycol), poly(aspartate), biomolecule, therapeutic moiety, diagnostic moiety, *etc.*) is also contemplated. In another exemplary embodiment, a therapeutic sugar moiety is conjugated to a linker arm and the sugar-linker arm is subsequently conjugated to a peptide

25   via a method of the invention.

Methods and chemistry for activation of water-soluble polymers and saccharides as well as methods for conjugating saccharides and polymers to various species are described in the literature. Commonly used methods for activation of polymers include activation of functional groups with cyanogen bromide, periodate, glutaraldehyde, biepoxides,

30   epichlorohydrin, divinylsulfone, carbodiimide, sulfonyl halides, trichlorotriazine, *etc.* (*see*, R. F. Taylor, (1991), PROTEIN IMMOBILISATION. FUNDAMENTALS AND APPLICATIONS, Marcel

Dekker, N.Y.; S. S. Wong, (1992), CHEMISTRY OF PROTEIN CONJUGATION AND
CROSSLINKING, CRC Press, Boca Raton; G. T. Hermanson *et al.*, (1993), IMMOBILIZED
AFFINITY LIGAND TECHNIQUES, Academic Press, N.Y.; Dunn, R.L., *et al.*, Eds. POLYMERIC
DRUGS AND DRUG DELIVERY SYSTEMS, ACS Symposium Series Vol. 469, American

5   Chemical Society, Washington, D.C. 1991).

       Routes for preparing reactive PEG molecules and forming conjugates using the
reactive molecules are known in the art. For example, U.S. Patent No. 5,672,662 discloses a
water soluble and isolatable conjugate of an active ester of a polymer acid selected from
linear or branched poly(alkylene oxides), poly(oxyethylated polyols), poly(olefinic alcohols),

10   and poly(acrylomorpholine), wherein the polymer has about 44 or more recurring units.

       U.S. Patent No. 6,376,604 sets forth a method for preparing a water-soluble 1-
benzotriazolylcarbonate ester of a water-soluble and non-peptidic polymer by reacting a
terminal hydroxyl of the polymer with di(1-benzotriazoyl)carbonate in an organic solvent.
The active ester is used to form conjugates with a biologically active agent such as a protein

15   or peptide.

       WO 99/45964 describes a conjugate comprising a biologically active agent and an
activated water soluble polymer comprising a polymer backbone having at least one terminus
linked to the polymer backbone through a stable linkage, wherein at least one terminus
comprises a branching moiety having proximal reactive groups linked to the branching

20   moiety, in which the biologically active agent is linked to at least one of the proximal reactive
groups. Other branched poly(ethylene glycols) are described in WO 96/21469, U.S. Patent
No. 5,932,462 describes a conjugate formed with a branched PEG molecule that includes a
branched terminus that includes reactive functional groups. The free reactive groups are
available to react with a biologically active species, such as a protein or peptide, forming

25   conjugates between the poly(ethylene glycol) and the biologically active species. U.S. Patent
No. 5,446,090 describes a bifunctional PEG linker and its use in forming conjugates having a
peptide at each of the PEG linker termini.

       Conjugates that include degradable PEG linkages are described in WO 99/34833; and
WO 99/14259, as well as in U.S. Patent No. 6,348,558. Such degradable linkages are

30   applicable in the present invention.

Although both reactive PEG derivatives and conjugates formed using the derivatives are known in the art, until the present invention, it was not recognized that a conjugate could be formed between PEG (or other polymer) and another species, such as a peptide or glycopeptide, through an intact glycosyl linking group.

5   Many water-soluble polymers are known to those of skill in the art and are useful in practicing the present invention. The term water-soluble polymer encompasses species such as saccharides (e.g., dextran, amylose, hyalouronic acid, poly(sialic acid), heparans, heparins, etc.); poly (amino acids), e.g., poly(glutamic acid); nucleic acids; synthetic polymers (e.g., poly(acrylic acid), poly(ethers), e.g., poly(ethylene glycol); peptides, proteins, and the like.

10   The present invention may be practiced with any water-soluble polymer with the sole limitation that the polymer must include a point at which the remainder of the conjugate can be attached.

Methods for activation of polymers can also be found in WO 94/17039, U.S. Pat. No. 5,324,844, WO 94/18247, WO 94/04193, U.S. Pat. No. 5,219,564, U.S. Pat. No. 5,122,614,

15   WO 90/13540, U.S. Pat. No. 5,281,698, and more WO 93/15189, and for conjugation between activated polymers and peptides, *e.g.* Coagulation Factor VIII (WO 94/15625), hemoglobin (WO 94/09027), oxygen carrying molecule (U.S. Pat. No. 4,412,989), ribonuclease and superoxide dismutase (Veronese *at al., App. Biochem. Biotech.* 11: 141-45 (1985)).

20   Preferred water-soluble polymers are those in which a substantial proportion of the polymer molecules in a sample of the polymer are of approximately the same molecular weight; such polymers are "homodisperse."

The present invention is further illustrated by reference to a poly(ethylene glycol) conjugate. Several reviews and monographs on the functionalization and conjugation of PEG

25   are available. *See,* for example, Harris, *Macronol. Chem. Phys.* C25: 325-373 (1985); Scouten, *Methods in Enzymology* 135: 30-65 (1987); Wong *et al., Enzyme Microb. Technol.* 14: 866-874 (1992); Delgado *et al., Critical Reviews in Therapeutic Drug Carrier Systems* 9: 249-304 (1992); Zalipsky, *Bioconjugate Chem.* 6: 150-165 (1995); and Bhadra, *et al., Pharmazie,* 57:5-29 (2002).

30   Poly(ethylene glycol) molecules suitable for use in the invention include, but are not limited to, those described by the following Formula 3:

Formula 3.

R= H, alkyl, benzyl, aryl, acetal, OHC-, $H_2N-CH_2CH_2-$, $HS-CH_2CH_2-$,

, -sugar-nucleotide, protein, methyl, ethyl;
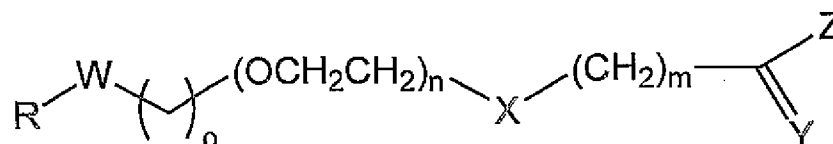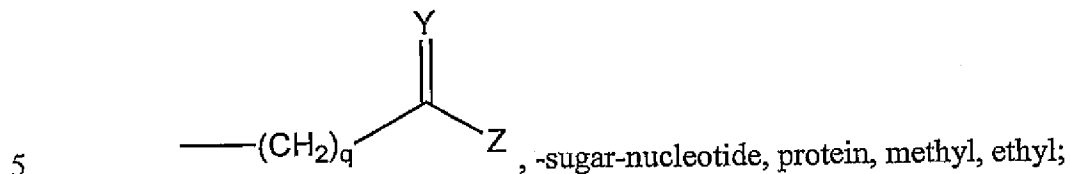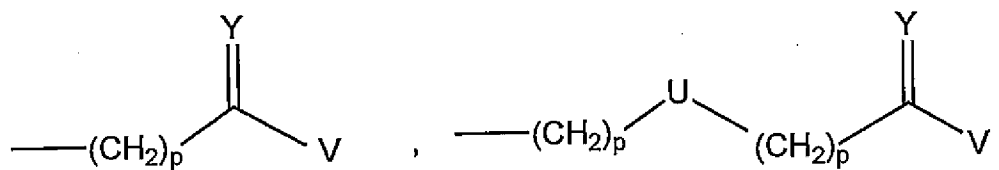
X, Y, W, U (independently selected) = O, S, NH, N-R';

R', R''' (independently selected) = alkyl, benzyl, aryl, alkyl aryl, pyridyl, substituted aryl, arylalkyl, acylaryl;

n = 1 to 2000;
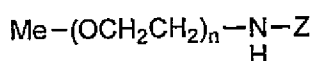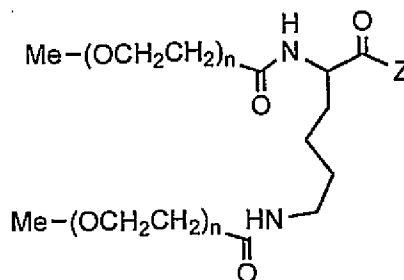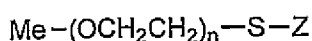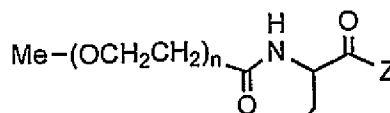
m, q, p (independently selected) = 0 to 20
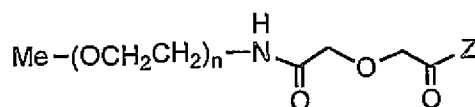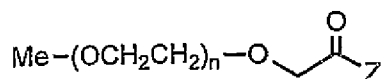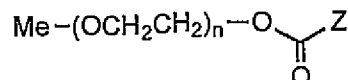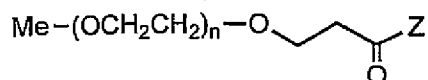
o = 0 to 20;

Z = HO, NH_2, halogen, S-R''', activated esters,

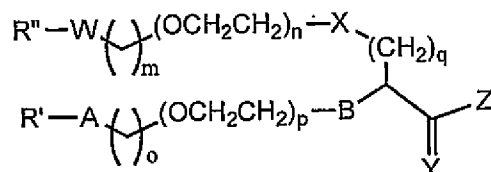-sugar-nucleotide, protein, imidazole, HOBT, tetrazole, halide; and

V = HO, NH_2, halogen, S-R''', activated esters, activated amides, -sugar-nucleotide, protein.

In preferred embodiments, the poly(ethylene glycol) molecule is selected from the following:

$$Me-(OCH_2CH_2)_n-O \diagdown \diagup \overset{O}{\underset{\|}{C}} \diagup Z$$

$$Me-(OCH_2CH_2)_n-O \diagup \overset{O}{\underset{\|}{C}} \diagup Z$$

$$Me-(OCH_2CH_2)_n-O \diagup \overset{O}{\underset{\|}{C}} \diagdown \underset{Z}{}$$

$$Me-(OCH_2CH_2)_n-\overset{H}{\underset{}{N}} \diagup \overset{O}{\underset{\|}{C}} \diagdown O \diagup \overset{O}{\underset{\|}{C}} \diagup Z$$

$$Me-(OCH_2CH_2)_n-O \diagdown \diagup \overset{O}{\underset{\|}{C}} \diagdown Z$$

$$Me-(OCH_2CH_2)_n-S-Z$$

$$Me-(OCH_2CH_2)_n-\underset{H}{N}-Z$$

$$Me-(OCH_2CH_2)_n \diagup \overset{H}{\underset{}{N}} \diagup \overset{O}{\underset{}{}} \diagdown Z$$
$$Me-(OCH_2CH_2)_n \diagdown \underset{\|}{\underset{O}{}} HN \diagup$$
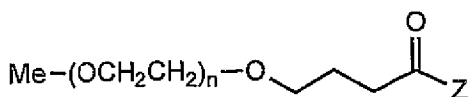
The poly(ethylene glycol) useful in forming the conjugate of the invention is either linear or branched. Branched poly(ethylene glycol) molecules suitable for use in the invention include, but are not limited to, those described by the following Formula:
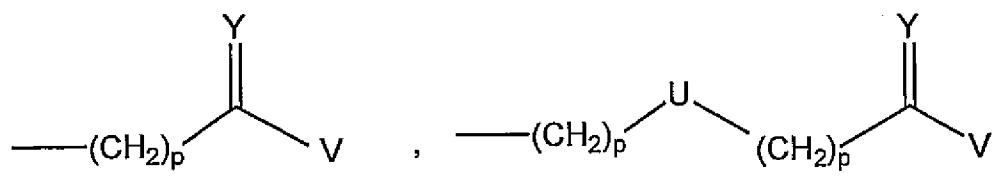
Formula 4:

$$R''-W \overset{}{\underset{m}{(\ )}} (OCH_2CH_2)_n-X \diagdown \underset{(CH_2)_q}{}$$
$$R'-A \overset{}{\underset{o}{(\ )}} (OCH_2CH_2)_p-B \diagup \overset{}{\underset{Y}{C}} \diagup Z$$

5

R', R'', R''' (independently selected) = H, alkyl, benzyl, aryl, acetal, OHC-, H_2N-CH_2CH_2-, HS-CH^2CH_2-, -$(CH_2)_q$CY-Z, -sugar-nucleotide, protein, methyl, ethyl, heteroaryl, acylalkyl, acylaryl, acylalkylaryl;

X, Y, W, A, B (independently selected) = O, S, NH, N-R', $(CH_2)_l$;

10   n, p (independently selected) = 1 to 2000;

m, q, o (independently selected) = 0 to 20;

Z = HO, NH_2, halogen, S-R''', activated esters,

$$---(CH_2)_p \diagup \overset{Y}{\underset{\|}{C}} \diagdown V \quad , \quad ---(CH_2)_p \diagup \overset{U}{} \diagdown (CH_2)_p \diagup \overset{Y}{\underset{\|}{C}} \diagdown V \quad ,$$

-sugar-nucleotide, protein;

15   V = HO, NH_2, halogen, S-R''', activated esters, activated amides, -sugar-nucleotide, protein.

The *in vivo* half-life, area under the curve, and/or residence time of therapeutic peptides can also be enhanced with water-soluble polymers such as polyethylene glycol (PEG) and polypropylene glycol (PPG). For example, chemical modification of proteins with PEG (PEGylation) increases their molecular size and decreases their surface- and functional

5   group-accessibility, each of which are dependent on the size of the PEG attached to the protein. This results in an improvement of plasma half-lives and in proteolytic-stability, and a decrease in immunogenicity and hepatic uptake (Chaffee *et al. J. Clin. Invest.* 89: 1643-1651 (1992); Pyatak *et al. Res. Commun. Chem. Pathol Pharmacol.* 29: 113-127 (1980)). PEGylation of interleukin-2 has been reported to increase its antitumor potency *in vivo* (Katre

10   *et al. Proc. Natl. Acad. Sci. USA.* 84: 1487-1491 (1987)) and PEGylation of a F(ab')2 derived from the monoclonal antibody A7 has improved its tumor localization (Kitamura *et al. Biochem. Biophys. Res. Commun.* 28: 1387-1394 (1990)).

In one preferred embodiment, the *in vivo* half-life of a peptide derivatized with a water-soluble polymer by a method of the invention is increased relevant to the *in vivo* half-

15   life of the non-derivatized peptide. In another preferred embodiment, the area under the curve of a peptide derivatized with a water-soluble polymer using a method of the invention is increased relevant to the area under the curve of the non-derivatized peptide. In another preferred embodiment, the residence time of a peptide derivatized with a water-soluble polymer using a method of the invention is increased relevant to the residence time of the

20   non-derivatized peptide. Techniques to determine the *in vivo* half-life, the area under the curve and the residence time are well known in the art. Descriptions of such techniques can be found in J.G. Wagner, 1993, Pharmacokinetics for the Pharmaceutical Scientist, Technomic Publishing Company, Inc. Lancaster PA.

The increase in peptide *in vivo* half-life is best expressed as a range of percent

25   increase in this quantity. The lower end of the range of percent increase is about 40%, about 60%, about 80%, about 100%, about 150% or about 200%. The upper end of the range is about 60%, about 80%, about 100%, about 150%, or more than about 250%.

In an exemplary embodiment, the present invention provides a PEGylated follicle stimulating hormone (Examples 23 and 24). In a further exemplary embodiment, the

30   invention provides a PEGylated transferrin (Example 42).

Other exemplary water-soluble polymers of use in the invention include, but are not limited to linear or branched poly(alkylene oxides), poly(oxyethylated polyols), poly(olefinic alcohols), and poly(acrylomorpholine), dextran, starch, poly(amino acids), etc.

b) Water-insoluble polymers

The conjugates of the invention may also include one or more water-insoluble polymers. This embodiment of the invention is illustrated by the use of the conjugate as a vehicle with which to deliver a therapeutic peptide in a controlled manner. Polymeric drug delivery systems are known in the art. See, for example, Dunn et al., Eds. POLYMERIC DRUGS AND DRUG DELIVERY SYSTEMS, ACS Symposium Series Vol. 469, American Chemical Society, Washington, D.C. 1991. Those of skill in the art will appreciate that substantially any known drug delivery system is applicable to the conjugates of the present invention.

Representative water-insoluble polymers include, but are not limited to, polyphosphazines, poly(vinyl alcohols), polyamides, polycarbonates, polyalkylenes, polyacrylamides, polyalkylene glycols, polyalkylene oxides, polyalkylene terephthalates, polyvinyl ethers, polyvinyl esters, polyvinyl halides, polyvinylpyrrolidone, polyglycolides, polysiloxanes, polyurethanes, poly(methyl methacrylate), poly(ethyl methacrylate), poly(butyl methacrylate), poly(isobutyl methacrylate), poly(hexyl methacrylate), poly(isodecyl methacrylate), poly(lauryl methacrylate), poly(phenyl methacrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), poly(octadecyl acrylate) polyethylene, polypropylene, poly(ethylene glycol), poly(ethylene oxide), poly (ethylene terephthalate), poly(vinyl acetate), polyvinyl chloride, polystyrene, polyvinyl pyrrolidone, pluronics and polyvinylphenol and copolymers thereof.

Synthetically modified natural polymers of use in conjugates of the invention include, but are not limited to, alkyl celluloses, hydroxyalkyl celluloses, cellulose ethers, cellulose esters, and nitrocelluloses. Particularly preferred members of the broad classes of synthetically modified natural polymers include, but are not limited to, methyl cellulose, ethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, hydroxybutyl methyl cellulose, cellulose acetate, cellulose propionate, cellulose acetate butyrate, cellulose acetate phthalate, carboxymethyl cellulose, cellulose triacetate, cellulose sulfate sodium salt, and polymers of acrylic and methacrylic esters and alginic acid.

These and the other polymers discussed herein can be readily obtained from commercial sources such as Sigma Chemical Co. (St. Louis, MO.), Polysciences (Warrenton, PA.), Aldrich (Milwaukee, WI.), Fluka (Ronkonkoma, NY), and BioRad (Richmond, CA), or else synthesized from monomers obtained from these suppliers using standard techniques.

5   Representative biodegradable polymers of use in the conjugates of the invention include, but are not limited to, polylactides, polyglycolides and copolymers thereof, poly(ethylene terephthalate), poly(butyric acid), poly(valeric acid), poly(lactide-co-caprolactone), poly(lactide-co-glycolide), polyanhydrides, polyorthoesters, blends and copolymers thereof. Of particular use are compositions that form gels, such as those

10   including collagen, pluronics and the like.

The polymers of use in the invention include "hybrid' polymers that include water-insoluble materials having within at least a portion of their structure, a bioresorbable molecule. An example of such a polymer is one that includes a water-insoluble copolymer, which has a bioresorbable region, a hydrophilic region and a plurality of crosslinkable

15   functional groups per polymer chain.

For purposes of the present invention, "water-insoluble materials" includes materials that are substantially insoluble in water or water-containing environments. Thus, although certain regions or segments of the copolymer may be hydrophilic or even water-soluble, the polymer molecule, as a whole, does not to any substantial measure dissolve in water.

20   For purposes of the present invention, the term "bioresorbable molecule" includes a region that is capable of being metabolized or broken down and resorbed and/or eliminated through normal excretory routes by the body. Such metabolites or break down products are preferably substantially non-toxic to the body.

The bioresorbable region may be either hydrophobic or hydrophilic, so long as the

25   copolymer composition as a whole is not rendered water-soluble. Thus, the bioresorbable region is selected based on the preference that the polymer, as a whole, remains water-insoluble. Accordingly, the relative properties, i.e., the kinds of functional groups contained by, and the relative proportions of the bioresorbable region, and the hydrophilic region are selected to ensure that useful bioresorbable compositions remain water-insoluble.

30   Exemplary resorbable polymers include, for example, synthetically produced resorbable block copolymers of poly(α-hydroxy-carboxylic acid)/poly(oxyalkylene, (see,

Cohn *et al.*, U.S. Patent No. 4,826,945). These copolymers are not crosslinked and are water-soluble so that the body can excrete the degraded block copolymer compositions. *See,* Younes *et al., J Biomed. Mater. Res.* **21**: 1301-1316 (1987); and Cohn *et al., J Biomed. Mater. Res.* **22**: 993-1009 (1988).

5   Presently preferred bioresorbable polymers include one or more components selected from poly(esters), poly(hydroxy acids), poly(lactones), poly(amides), poly(ester-amides), poly (amino acids), poly(anhydrides), poly(orthoesters), poly(carbonates), poly(phosphazines), poly(phosphoesters), poly(thioesters), polysaccharides and mixtures thereof. More preferably still, the biosresorbable polymer includes a poly(hydroxy) acid

10   component. Of the poly(hydroxy) acids, polylactic acid, polyglycolic acid, polycaproic acid, polybutyric acid, polyvaleric acid and copolymers and mixtures thereof are preferred.

In addition to forming fragments that are absorbed *in vivo* ("bioresorbed"), preferred polymeric coatings for use in the methods of the invention can also form an excretable and/or metabolizable fragment.

15   Higher order copolymers can also be used in the present invention. For example, Casey *et al.*, U.S. Patent No. 4,438,253, which issued on March 20, 1984, discloses tri-block copolymers produced from the transesterification of poly(glycolic acid) and an hydroxyl-ended poly(alkylene glycol). Such compositions are disclosed for use as resorbable monofilament sutures. The flexibility of such compositions is controlled by the incorporation

20   of an aromatic orthocarbonate, such as tetra-p-tolyl orthocarbonate into the copolymer structure.

Other coatings based on lactic and/or glycolic acids can also be utilized. For example, Spinu, U.S. Patent No. 5,202,413, which issued on April 13, 1993, discloses biodegradable multi-block copolymers having sequentially ordered blocks of polylactide and/or

25   polyglycolide produced by ring-opening polymerization of lactide and/or glycolide onto either an oligomeric diol or a diamine residue followed by chain extension with a di-functional compound, such as, a diisocyanate, diacylchloride or dichlorosilane.

Bioresorbable regions of coatings useful in the present invention can be designed to be hydrolytically and/or enzymatically cleavable. For purposes of the present invention,

30   "hydrolytically cleavable" refers to the susceptibility of the copolymer, especially the bioresorbable region, to hydrolysis in water or a water-containing environment. Similarly,

"enzymatically cleavable" as used herein refers to the susceptibility of the copolymer, especially the bioresorbable region, to cleavage by endogenous or exogenous enzymes.

When placed within the body, the hydrophilic region can be processed into excretable and/or metabolizable fragments. Thus, the hydrophilic region can include, for example,

5   polyethers, polyalkylene oxides, polyols, poly(vinyl pyrrolidine), poly(vinyl alcohol), poly(alkyl oxazolines), polysaccharides, carbohydrates, peptides, proteins and copolymers and mixtures thereof. Furthermore, the hydrophilic region can also be, for example, a poly(alkylene) oxide. Such poly(alkylene) oxides can include, for example, poly(ethylene) oxide, poly(propylene) oxide and mixtures and copolymers thereof.

10   Polymers that are components of hydrogels are also useful in the present invention. Hydrogels are polymeric materials that are capable of absorbing relatively large quantities of water. Examples of hydrogel forming compounds include, but are not limited to, polyacrylic acids, sodium carboxymethylcellulose, polyvinyl alcohol, polyvinyl pyrrolidine, gelatin, carrageenan and other polysaccharides, hydroxyethylenemethacrylic acid (HEMA), as well as

15   derivatives thereof, and the like. Hydrogels can be produced that are stable, biodegradable and bioresorbable. Moreover, hydrogel compositions can include subunits that exhibit one or more of these properties.

Bio-compatible hydrogel compositions whose integrity can be controlled through crosslinking are known and are presently preferred for use in the methods of the invention.

20   For example, Hubbell et al., U.S. Patent Nos. 5,410,016, which issued on April 25, 1995 and 5,529,914, which issued on June 25, 1996, disclose water-soluble systems, which are crosslinked block copolymers having a water-soluble central block segment sandwiched between two hydrolytically labile extensions. Such copolymers are further end-capped with photopolymerizable acrylate functionalities. When crosslinked, these systems become

25   hydrogels. The water soluble central block of such copolymers can include poly(ethylene glycol); whereas, the hydrolytically labile extensions can be a poly(α-hydroxy acid), such as polyglycolic acid or polylactic acid. See, Sawhney et al., Macromolecules 26: 581-587 (1993).

In another preferred embodiment, the gel is a thermoreversible gel. Thermoreversible

30   gels including components, such as pluronics, collagen, gelatin, hyalouronic acid,

polysaccharides, polyurethane hydrogel, polyurethane-urea hydrogel and combinations thereof are presently preferred.

In yet another exemplary embodiment, the conjugate of the invention includes a component of a liposome. Liposomes can be prepared according to methods known to those

5   skilled in the art, for example, as described in Eppstein *et al.*, U.S. Patent No. 4,522,811, which issued on June 11, 1985. For example, liposome formulations may be prepared by dissolving appropriate lipid(s) (such as stearoyl phosphatidyl ethanolamine, stearoyl phosphatidyl choline, arachadoyl phosphatidyl choline, and cholesterol) in an inorganic solvent that is then evaporated, leaving behind a thin film of dried lipid on the surface of the

10   container. An aqueous solution of the active compound or its pharmaceutically acceptable salt is then introduced into the container. The container is then swirled by hand to free lipid material from the sides of the container and to disperse lipid aggregates, thereby forming the liposomal suspension.

The above-recited microparticles and methods of preparing the microparticles are

15   offered by way of example and they are not intended to define the scope of microparticles of use in the present invention. It will be apparent to those of skill in the art that an array of microparticles, fabricated by different methods, are of use in the present invention.

c) Biomolecules

In another preferred embodiment, the modified sugar bears a biomolecule. In still

20   further preferred embodiments, the biomolecule is a functional protein, enzyme, antigen, antibody, peptide, nucleic acid (*e.g.*, single nucleotides or nucleosides, oligonucleotides, polynucleotides and single- and higher-stranded nucleic acids), lectin, receptor or a combination thereof.

Some preferred biomolecules are essentially non-fluorescent, or emit such a minimal

25   amount of fluorescence that they are inappropriate for use as a fluorescent marker in an assay. Other biomolecules may be fluorescent. The use of an otherwise naturally occurring sugar that is modified by covalent attachment of another entity (*e.g.*, PEG, biomolecule, therapeutic moiety, diagnostic moiety, *etc.*) is appropriate. In an exemplary embodiment, a sugar moiety, which is a biomolecule, is conjugated to a linker arm and the sugar-linker arm cassette is

30   subsequently conjugated to a peptide via a method of the invention.

Biomolecules useful in practicing the present invention can be derived from any source. The biomolecules can be isolated from natural sources or they can be produced by synthetic methods. Peptides can be natural peptides or mutated peptides. Mutations can be effected by chemical mutagenesis, site-directed mutagenesis or other means of inducing

5   mutations known to those of skill in the art. Peptides useful in practicing the instant invention include, for example, enzymes, antigens, antibodies and receptors. Antibodies can be either polyclonal or monoclonal; either intact or fragments. The peptides are optionally the products of a program of directed evolution.

Both naturally derived and synthetic peptides and nucleic acids are of use in

10   conjunction with the present invention; these molecules can be attached to a sugar residue component or a crosslinking agent by any available reactive group. For example, peptides can be attached through a reactive amine, carboxyl, sulfhydryl, or hydroxyl group. The reactive group can reside at a peptide terminus or at a site internal to the peptide chain. Nucleic acids can be attached through a reactive group on a base (*e.g.*, exocyclic amine) or an

15   available hydroxyl group on a sugar moiety (*e.g.*, 3'- or 5'-hydroxyl). The peptide and nucleic acid chains can be further derivatized at one or more sites to allow for the attachment of appropriate reactive groups onto the chain. *See*, Chrisey *et al. Nucleic Acids Res.* 24: 3031-3039 (1996).

In a further preferred embodiment, the biomolecule is selected to direct the peptide

20   modified by the methods of the invention to a specific tissue, thereby enhancing the delivery of the peptide to that tissue relative to the amount of underivatized peptide that is delivered to the tissue. In a still further preferred embodiment, the amount of derivatized peptide delivered to a specific tissue within a selected time period is enhanced by derivatization by at least about 20%, more preferably, at least about 40%, and more preferably still, at least about

25   100%. Presently, preferred biomolecules for targeting applications include antibodies, hormones and ligands for cell-surface receptors. Exemplary targeting biomolecules include, but are not limited to, an antibody specific for the transferrin receptor for delivery of the molecule to the brain (Penichet et al., 1999, J. Immunol. 163:4421-4426; Pardridge, 2002, Adv. Exp. Med. Biol. 513:397-430), a peptide that recognizes the vasculature of the prostate

30   (Arap et al., 2002, PNAS 99:1527-1531), and an antibody specific for lung caveolae (McIntosh et al., 2002, PNAS 99:1996-2001).

In a presently preferred embodiment, the modifying group is a protein. In an
exemplary embodiment, the protein is an interferon. The interferons are antiviral
glycoproteins that, in humans, are secreted by human primary fibroblasts after induction with
virus or double-stranded RNA. Interferons are of interest as therapeutics, e.g., antivirals and
5   treatment of multiple sclerosis. For references discussing interferon-β, see, e.g., Yu, et al., J.
Neuroimmunol., 64(1):91-100 (1996); Schmidt, J., J. Neurosci. Res., 65(1):59-67 (2001);
Wender, et al., Folia Neuropathol., 39(2):91-93 (2001); Martin, et al., Springer Semin.
Immunopathol., 18(1):1-24 (1996); Takane, et al., J. Pharmacol. Exp. Ther., 294(2):746-752
(2000); Sburlati, et al., Biotechnol. Prog., 14:189-192 (1998); Dodd, et al., Biochimica et
10   Biophysica Acta, 787:183-187 (1984); Edelbaum, et al., J. Interferon Res., 12:449-453
(1992); Conradt, et al., J. Biol. Chem., 262(30):14600-14605 (1987); Civas, et al., Eur. J.
Biochem., 173:311-316 (1988); Demolder, et al., J. Biotechnol., 32:179-189 (1994); Sedmak,
et al., J. Interferon Res., 9(Suppl 1):S61-S65 (1989); Kagawa, et al., J. Biol. Chem.,
263(33):17508-17515 (1988); Hershenson, et al., U.S. Patent No. 4,894,330; Jayaram, et al.,
15   J. Interferon Res., 3(2):177-180 (1983); Menge, et al., Develop. Biol. Standard., 66:391-401
(1987); Vonk, et al., J. Interferon Res., 3(2):169-175 (1983); and Adolf, et al., J. Interferon
Res., 10:255-267 (1990). For references relevant to interferon-α, see, Asano, et al., Eur. J.
Cancer, 27(Suppl 4):S21-S25 (1991); Nagy, et al., Anticancer Research, 8(3):467-470
(1988); Dron, et al., J. Biol. Regul. Homeost. Agents, 3(1):13-19 (1989); Habib, et al., Am.
20   Surg., 67(3):257-260 (3/2001); and Sugyiama, et al., Eur. J. Biochem., 217:921-927 (1993).

In an exemplary interferon conjugate, interferon β is conjugated to a second peptide
via a linker arm. The linker arm includes an intact glycosyl linking group through which it is
attached to the second peptide via a method of the invention. The linker arm also optionally
includes a second intact glycosyl linking group, through which it is attached to the interferon.

25   In another exemplary embodiment, the invention provides a conjugate of follicle
stimulating hormone (FSH). FSH is a glycoprotein hormone. See, for example, Saneyoshi,
et al., Biol. Reprod., 65:1686-1690 (2001); Hakola, et al., J. Endocrinol., 158:441-448
(1998); Stanton, et al., Mol. Cell. Endocrinol., 125:133-141 (1996); Walton, et al., J. Clin.
Endocrinol. Metab., 86(8):3675-3685 (08/2001); Ulloa-Aguirre, et al., Endocrine, 11(3):205-
30   215 (12/1999); Castro-Fernández, et al.I, J. Clin. Endocrinol. Matab., 85(12):4603-4610
(2000); Prevost, Rebecca R., Pharmacotherapy, 18(5):1001-1010 (1998); Linskens, et al.,

The FASEB Journal, 13:639-645 (04/1999); Butnev, *et al.*, *Biol. Reprod.*, 58:458-469 (1998); Muyan, *et al.*, *Mol. Endo.*, 12(5):766-772 (1998); Min, *et al.*, *Endo. J.*, 43(5):585-593 (1996); Boime, *et al.*, *Recent Progress in Hormone Research*, 34:271-289 (1999); and Rafferty, *et al.*, *J. Endo.*, 145:527-533 (1995). The FSH conjugate can be formed in a manner similar to that

5   described for interferon.

In yet another exemplary embodiment, the conjugate includes erythropoietin (EPO). EPO is known to mediate response to hypoxia and to stimulate the production of red blood cells. For pertinent references, *see*, Cerami, *et al.*, *Seminars in Oncology*, 28(2)(Suppl 8):66-70 (04/2001). An exemplary EPO conjugate is formed analogously to the conjugate of

10   interferon.

In a further exemplary embodiment, the invention provides a conjugate of human granulocyte colony stimulating factor (G-CSF). G-CSF is a glycoprotein that stimulates proliferation, differentiation and activation of neutropoietic progenitor cells into functionally mature neutrophils. Injected G-CSF is known to be rapidly cleared from the body. *See*, for

15   example, Nohynek, *et al.*, *Cancer Chemother. Pharmacol.*, 39:259-266 (1997); Lord, *et al.*, *Clinical Cancer Research*, 7(7):2085-2090 (07/2001); Rotondaro, *et al.*, *Molecular Biotechnology*, 11(2):117-128 (1999); and Bönig, *et al.*, *Bone Marrow Transplantation*, 28:259-264 (2001). An exemplary conjugate of G-CSF is prepared as discussed above for the conjugate of the interferons. One of skill in the art will appreciate that many other

20   proteins may be conjugated to interferon using the methods and compositions of the invention, including but not limited to, the peptides listed in Tables 7 and 8 (presented elsewhere herein) and Figure 28, and in Figures 29-57, where individual modification schemes are presented.

In still a further exemplary embodiment, there is provided a conjugate with biotin.

25   Thus, for example, a selectively biotinylated peptide is elaborated by the attachment of an avidin or streptavidin moiety bearing one or more modifying groups.

In a further preferred embodiment, the biomolecule is selected to direct the peptide modified by the methods of the invention to a specific intracellular compartment, thereby enhancing the delivery of the peptide to that intracellular compartment relative to the amount

30   of underivatized peptide that is delivered to the tissue. In a still further preferred embodiment, the amount of derivatized peptide delivered to a specific intracellular

compartment within a selected time period is enhanced by derivatization by at least about 20%, more preferably, at least about 40%, and more preferably still, at least about 100%. In another particularly preferred embodiment, the biomolecule is linked to the peptide by a cleavable linker that can hydrolyze once internalized. Presently, preferred biomolecules for

5   intracellular targeting applications include transferrin, lactotransferrin (lactoferrin), melanotransferrin (p97), ceruloplasmin, and divalent cation transporter, as well as antibodies directed against specific vascular targets. Contemplated linkages include, but are not limited to, protein-sugar-linker-sugar-protein, protein-sugar-linker-protein and multivalent forms thereof, and protein-sugar-linker-drug where the drug includes small molecules, peptides,

10   lipids, among others.

       Site-specific and target-oriented delivery of therapeutic agents is desirable for the purpose of treating a wide variety of human diseases, such as different types of malignancies and certain neurological disorders. Such procedures are accompanied by fewer side effects and a higher efficiacy of drug. Various principles have been relied on in designing these

15   delivery systems. For a review, see Garnett, *Advanced Drug Delivery Reviews* 53:171-216 (2001).
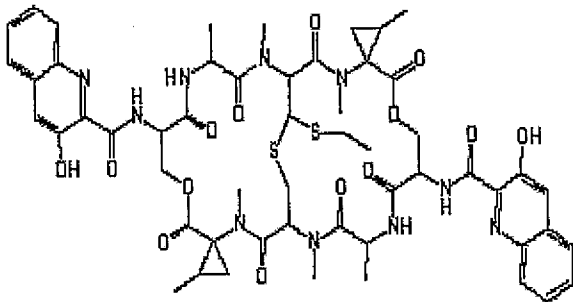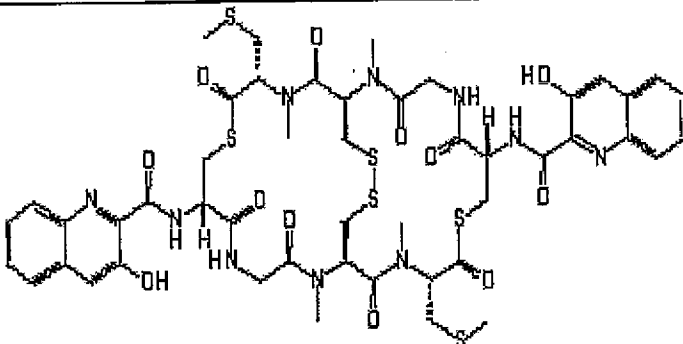
       One important consideration in designing a drug delivery system to target tissues specifically. The discovery of tumor surface antigens has made it possible to develop therapeutic approaches where tumor cells displaying definable surface antigens are

20   specifically targeted and killed. There are three main classes of therapeutic monoclonal antibodies (antibody) that have demonstrated effectiveness in human clinical trials in treating malignancies: (1) unconjugated MAb, which either directly induces growth inhibition and/or apoptosis, or indirectly activates host defense mechanisms to mediate antitumor cytotoxicity; (2) drug-conjugated MAb, which preferentially delivers a potent cytotoxic toxin to the tumor

25   cells and therefore minimizes the systemic cytotoxicity commonly associated with conventional chemotherapy; and (3) radioisotope-conjugated MAb, which delivers a sterilizing dose of radiation to the tumor. See review by Reff et al., *Cancer Control* 9:152-166 (2002).

       In order to arm MAbs with the power to kill malignant cells, the MAbs can be

30   connected to a toxin, which may be obtained from a plant, bacterial, or fungal source, to form chimeric proteins called immunotoxins. Frequently used plant toxins are divided into two

classes: (1) holotoxins (or class II ribosome inactivating proteins), such as ricin, abrin, mistletoe lectin, and modeccin, and (2) hemitoxins (class I ribosome inactivating proteins), such as pokeweed antiviral protein (PAP), saporin, Bryodin 1, bouganin, and gelonin. Commonly used bacterial toxins include diphtheria toxin (DT) and Pseudomonas exotoxin

5   (PE). Kreitman, *Current Pharmaceutical Biotechnology* 2:313-325 (2001). Other toxins contemplated for use with the present invention include, but are not limited to, those in Table 2.

Table 2. Toxins.

Chemical Structure				
Toxin Name/ Source/ Alternate ID	CAS RN / Analogs	Indication/ Toxicity	Mechanism	Activity (IC50 nM); Tumor Type
SW-163E/ *Streptomyces* sp SNA 15896/ SW-163E	260794-24-9; 260794-25-0/ SW-163C; SW-163A; SW-163B	Cancer and Antibacterial/ low toxicity (mice ip)	not reported	0.3 P388 0.2 A2780 0.4 KB 1.6 colon 1.3 HL-60
Thiocoraline/ *Micromonospora marina* (actinomycete)	173046-02-1	Breast Cancer; Melanoma; Non-small lung cancer / not reported	DNA Polymerase alpha inhibitor (blocks cell progression from G1 to	lung, colon, CNS melanoma

S)
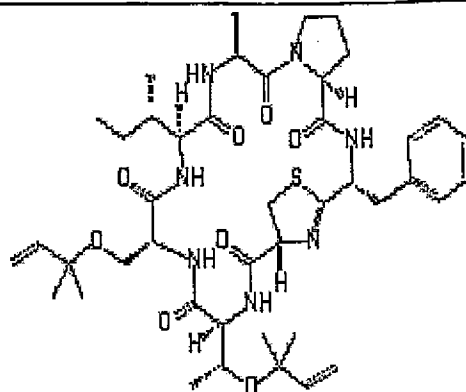
| Trunkamide A[1]/ *Lissoclinum* sp (aascidian) | 181758-83-8 | Cancer/ not reported | not reported | cell culture (IC50 in micrograms/mL); 0.5 P388; 0.5 A549; 0.5 HT-29; 1.0 MEL-28 |

| Palauamine[2]/ *Stylotella agminata* (sponge) | 148717-58-2 | Lung cancer/ LD50 (i.p. in mice) is 13 mg/Kg | not reported | cell culture (IC50 in micrograms/mL); 0.1 P388 0.2 A549 (lung) 2 HT-29 (colon) 10 KB |

| Halichondrin B/ *Halichondria Okadai, Axinell Carteri and Phankell carteri* (sponges)/ NSC-609385 | 103614-76-2/ isohomohalic hondrin B | cancer/ myelotoxicity dose limiting (dogs, rats) | antitubulin; cell cycle inhibitor (inhibits GTP binding to tubulin) | NCI tumor panel; GI(50) from 50 nM to 0.1 nM; LC50's from 40 μM to 0.1 nM (many 0.1 to 25 nM) |
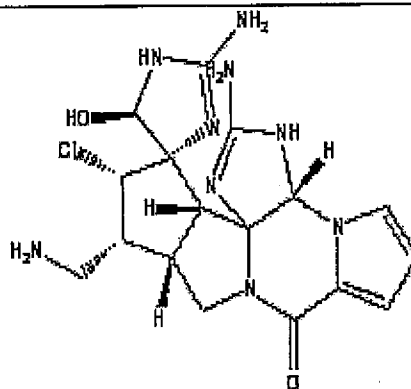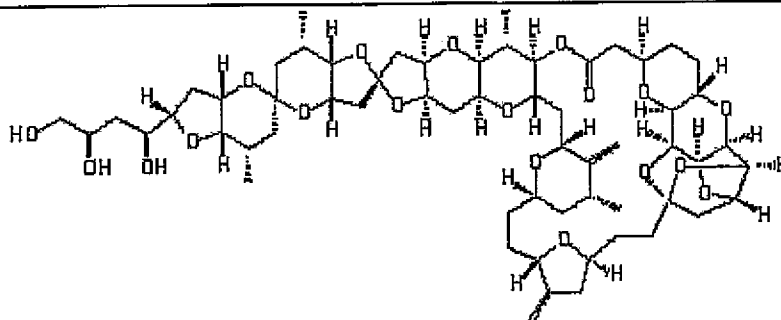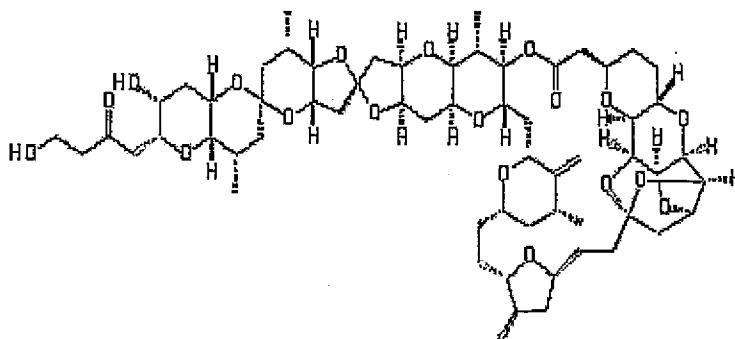
Isohomo-halichondrin B/ *Halichondria Okadai, Axinell Carteri and Phankell carteri* (sponges)/ NSC-650467	157078-48-3/ halichondrin B	melanoma, lung, CNS, colon, ovary/ not reported	antitubulin; cell cycle inhibitor (inhibits GTP binding to tubulin)	IC50's in 0.1 nM range (NCI tumor panel)

Halichondrin B analogs/ semi-synthetic starting from *Halichondria Okadai, Axinell Carteri and Phankell carteri* (sponges)/ ER-076349; ER-086526; B-1793; E-7389	253128-15-3/ ER-076349; ER-086526; B-1793; E-7389	solid tumors/ not reported	tubulin binding agent; disruption of mitotic spindles	cell culture (not reported); animal models active (tumor regression observed) in lymphoma, colon (multi-drug resistant).

NK-130119/ *Streptomyces*	132707-68-7	antifungal and anticancer/	not reported	25 ng/mL colon 8.5 ng/mL lung

bottropensis/ NK-130119		not reported		
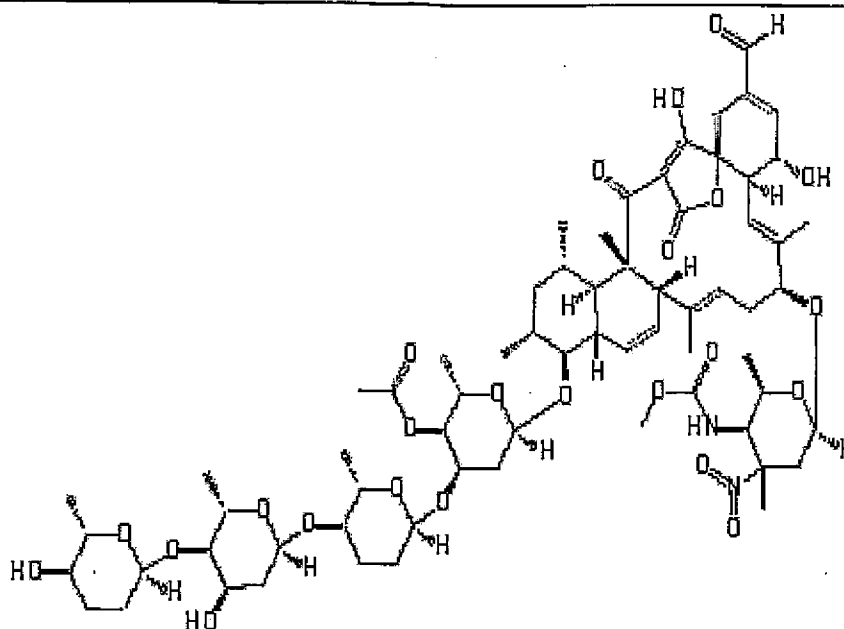
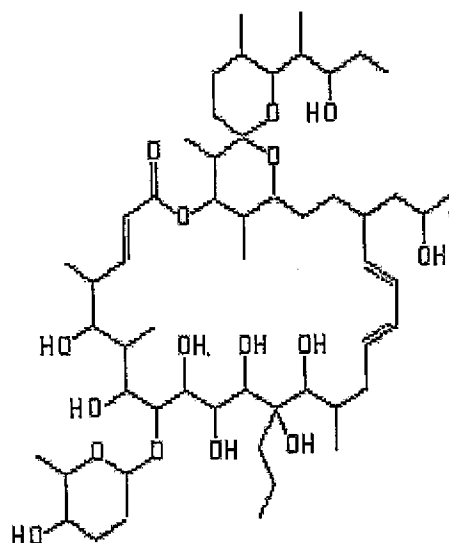| Tetrocarcin A/ not reported/ KF-67544	73666-84-9/ analogs are reported	cancer/ not reported	inhibits the anti-apoptotic functino of Bcl2	not reported
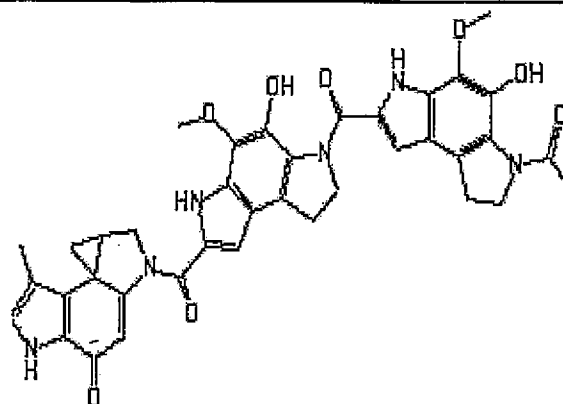
Gilvusmycin/ *Streptomyces* QM16	195052-09-6	cancer/ not reported	not reported	IC50's in ng/mL: 0.08 P388 0.86 K562 (CML) 0.72 A431 (EC) 0.75 MKN28 (GI); (for all < 1 nM)

IB-96212/
marine actinomycete/
IB-96212

220858-11-7/   Cancer and   not reported   IC50's in ng/mL:
IB-96212;   Antibacterial/   0.1   P388
IB-98214;   not reported
IB-97227
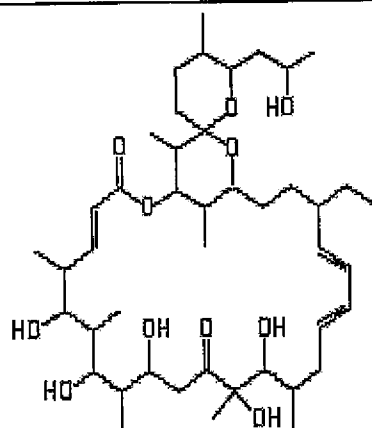
BE-56384[3]/
Streptomyces Sp./
BE-56384

207570-04-5   cancer/   not reported   IC50's in ng/mL:
                not reported   0.1   P388
                                                   0.29   colon 26
                                                   34   DLD-1
                                                   0.12 PC-13
                                                   0.12 MKM-45
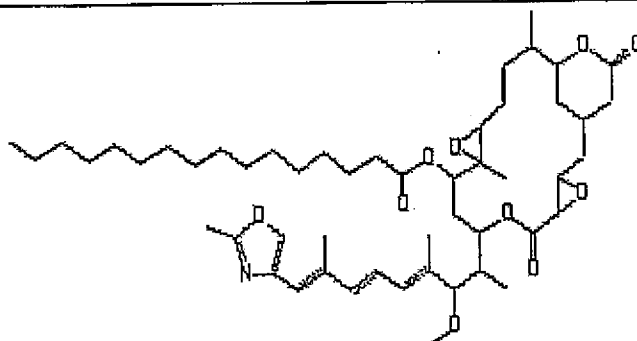
Palmitoylrhizoxin/   135819-69-1/   cancer/   tubulin   not reported

semi-synthetic; *Rhizopus chinensis*	Analog of rhizoxin	binds LDL; less cytotoxic than rhizoxin	binding agent (cell cycle inhibitor)
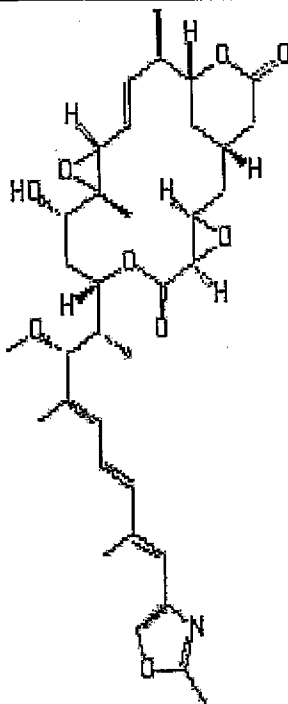
Rhizoxin/ *Rhizopus chinensis/* WF-1360; NSC-332598; FR-900216	95917-95-6; 90996-54-6	melanoma, lung, CNS, colon, ovary, renal, breast, head and neck/ Rapid Drug clearance; High AUC correlates with high toxicity	tubulin binding agent (cell cycle inhibitor)	NCI tumor panel (NSC 332598); log GI50's: 50 nM to 50 fM; log LC50's: 50 μM to 0.5 nM (several cell lines at 50 fM).
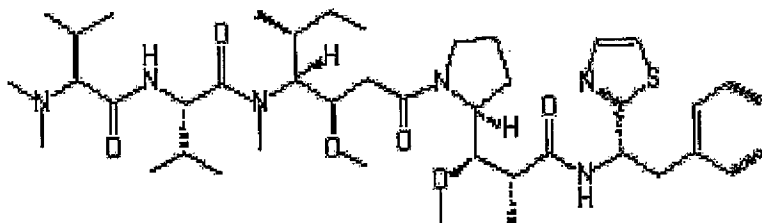
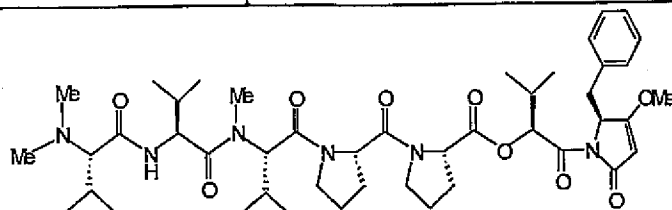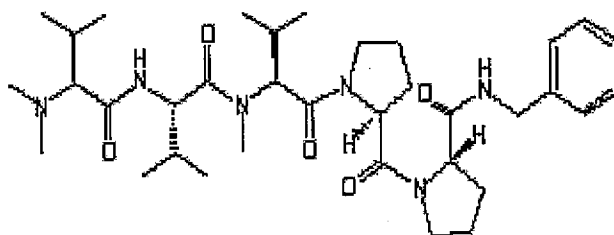| **Dolastatin-10/** *Dolabella auricularia* (sea hare)/ NSC-376128	110417-88-4/ other Dolistatins (ie. 15) and analogs	prostate, melanoma, leukemia/ myelotoxicity (at greater than 0.3 pM)	tubulin binding (tubulin aggregation)	NCI tumor panel (60 cell line; GI50); 25 nM to 1 pM (most < 1 nM) (three cell lines μM)

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soblidotin/ synthetic/ TZT-1027; auristatin PE	149606-27-9/ analogs prepared	cancer (pancreas, esophageal colon, breast, lung, etc) / MTD was 1.8 mg/Kg (IV); toxicity not reported	tubulin binding agent	cell culture: colon, melanoma, M5076 tumors, P388 with 75-85% inhibition (dose not reported)

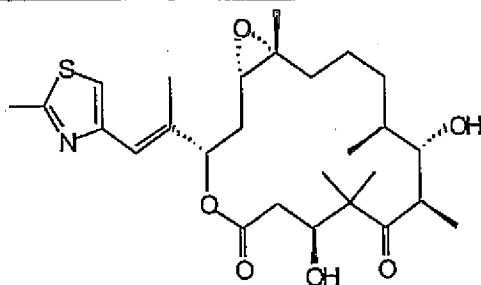| Dolastatin-15/ Dolabella auricularia (sea hare)	not reported/ other Dolistatins (ie. 15) and analogs	cancer/ not reported	Tubulin binding (tubuline aggregation)	NCI tumor panel (60 cell line; GI50); 25 nM to 39 pM (most < 1 nM) (one cell line 2.5 µM); most active in breast

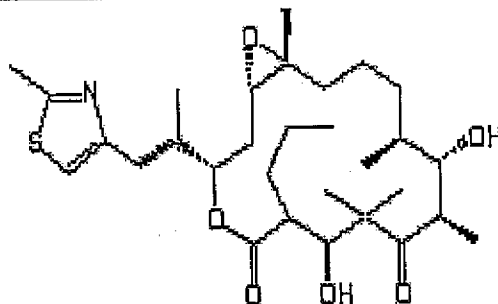| Cemadotin[4]/ Synthetic; Parent Dolastatin-15 was isolated from Dolabella auricularia (sea hare)/ LU-103793; NSC D-669356	1159776-69-9/ many analogs	melanoma/ hypertension, myocardial ischemia and myelosuppression were dose-limiting toxicities.	tubulin binding (tubulin aggregation)	NCI tumor panel (NCS D-669356); active in breast, ovary, endometrial, sarcomas and drug resistant cell lines. Data not public.
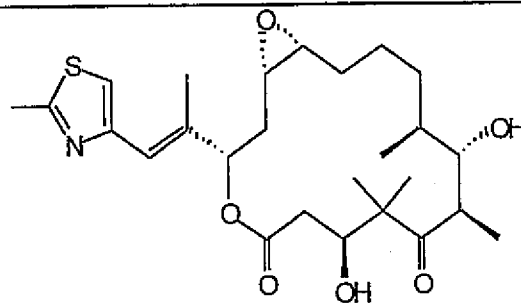
Epothilone A/	not reported/	cancer/	tubulin	IC50's of;

| Synthetic or isolated from *Sorangium cellulosum* (myxococcales) strain So ce90) | many analogs | not reported | | binding (tubulin polymeriza-tion) | 1.5 nM MCF-7 (breast) 27.1 nM MCF-7/ADR 2.1 nM KB-31 (melanoma) 3.2 nM HCT-116 |

| Epothilone B/ Synthetic or isolated from *Sorangium cellulosum* (myxococcales) strain So ce90) / EPO-906 | 152044054-7/ many analogs | Solid tumors (breast, ovarian, etc)/ well tolerated; t1/2 of 2.5 hrs; partial responses (phase I); diarrhea major side effect. | | tubulin binding (tubulin polymeriza-tion) | IC50's of; 0.18 nM MCF-7 (breast) 2.92 nM MCF-7/ADR 0.19 nM KB-31 (melanoma) 0.42 nM HCT-116; broad activity reported |

| Epothilone Analog / Synthetic or semi-synthetic; Original lead, Epothilone A, isolated from *Sorangium cellulosum* (myxococcales) strain So ce90)/ ZK-EPO | not reported / hundreds of analogs | cancer/ not reported | | tubulin binding (tubulin polymeriza-tion) | IC50's of 0.30 to 1.80 nM in various tumor cell lines; active in drug resistant cell lines |

| Epothilone D / Epothilone D, isolated from *Sorangium cellulosum* | 189452-10-9/ many analogs | Solid tumors (breast, ovarian, etc)/ emesis and anemia; t1/2 of 5-10 hrs. | tubulin binding (tubulin polymeriza- | NCI tumor panel (NSC-703147; IC50); 0.19 nM KB-31 (melanoma) |
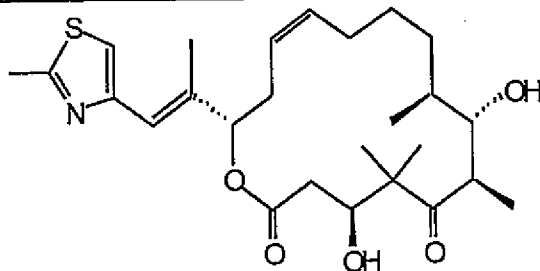
(myxococcales) strain So ce90)/ KOS-862			tion)	0.42 nM HCT-116; broad activity reported

Structure Not Identified

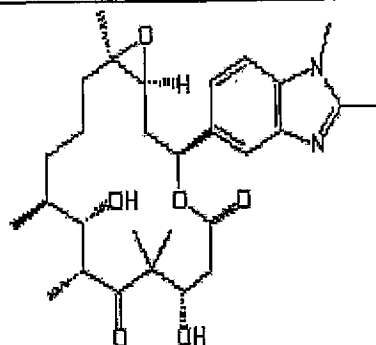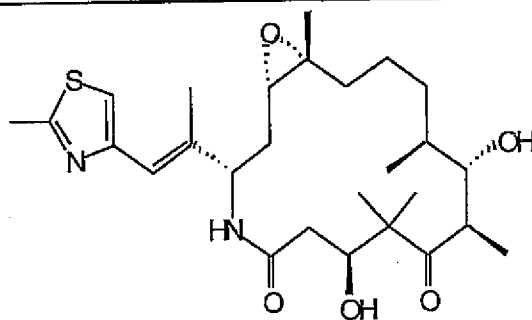|				
Epothilone D analog [5]/ Synthetic or semi-synthetic; Original lead, Epothilone D, isolated from *Sorangium cellulosum* (myxococcales) strain So ce90)/ KOS-166-24	189453-10-9/ hundreds of analogs	Solid tumors; not reported	tubulin binding (tubulin polymeriza-tion)	not reported

Epothilone Analog / Synthetic; Original lead, Epothilone A, isolated from *Sorangium cellulosum* (myxococcales) strain So ce90)/ CGP-85715	not reported/ hundreds of analogs	cancer; not reported	tubulin binding (tubulin polymeriza-tion)	not reported

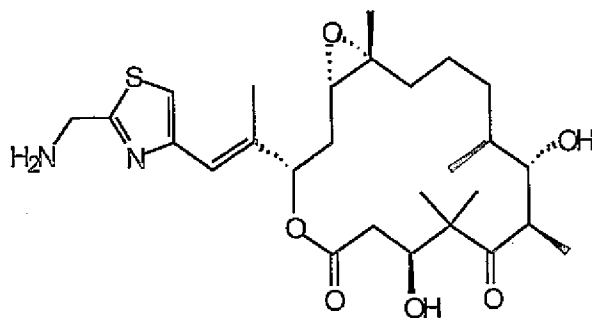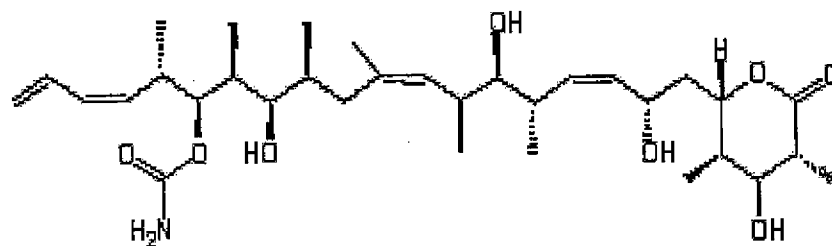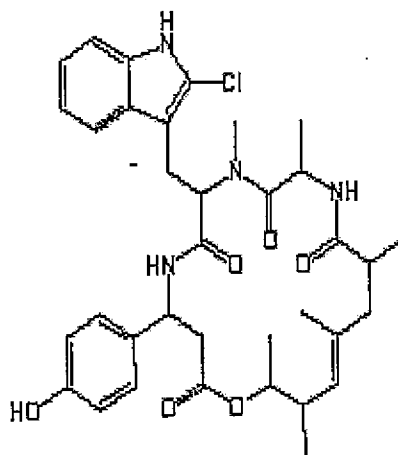|				
Epothilone Analog/ Synthetic or semi-synthetic; Original lead, Epothilone B, isolated from *Sorangium cellulosum* (myxococcales) strain So ce90)/ BMS-247550	219989-84-1/ hundreds of analogs	non-small cell Lung, breast, stomach tumor (objective responses in breast ovarian and lung)/ sever toxicity (fatigue, anorexia, nauseas, vomiting, neuropathy myalgia)	tubulin binding (tubulin polymerizati on)	NCI tumor Panel (NSC-710428 & NSC-710468); 8-32 nM (NCI data not available)

| Epothilone Analog / Synthetic or semi-synthetic; Original lead, Epothilone B, isolated from *Sorangium cellulosum* (myxococcales) strain So ce90)/ BMS-310705 | not reported/ hundreds of analogs | advanced cancers/ adverse events (diarrhea, nausea, vomiting , fatigue, neutropenia); t1/2 of 3.5 hrs; improved water solubility to BMS 247550. | tubulin binding (tubulin polymeriza-tion) | broad activity with IC50's of 0.7 to 10 nM |

| Discodermolide / synthetic; orginally isolated from *Discodermia dissoluta* (deep water sponge); rare compound (7 mg per 0.5 Kg sponge/ XAA-296 | 127943-53-7/ analogs less potent | solid tumors/ not reported; 100-fold increase in water solubility over taxol | tubulin stabilizing agent (similar to taxol) | Broad activity (A549-nsclung, prostate, P388, ovarian with IC50's about 10 nM) including multi-drug resistant cell lines; |

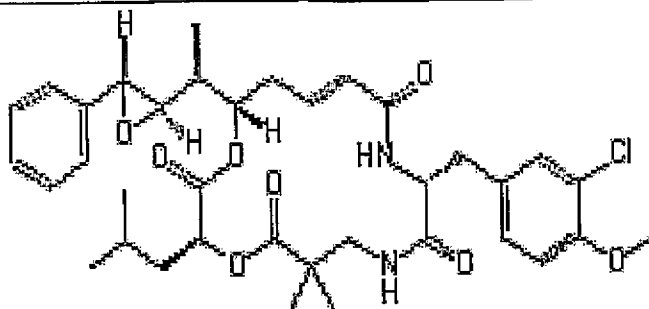| Chondramide D/ | 172430-63-6 | cancer/ | | tubulin | 5 nM A-549 |

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not reported	not reported	binding agent; actin polymeriza-tion inhibitor	(epidermoid carcinoma) 15 nM A-498 (kidney) 14 nM A549 (lung) 5 nM SK-OV-3 (ovary) 3 nM U-937 (lymphoma)

Cryptophycin analogs (including 52, 55 and others)[6]/ Nostoc sp GSV 224 (blue-green algae) isolated Cryptophycin 1./ LY-355703; Ly-355702; NSC-667642	204990-60-3 and 186256-67-7/ many potent analogs prepared at Lilly	solid tumors, colon cancer/ Phase II studies halted because of severe toxicity with one death resulting from drug;	tubulin polymeriza-tion inhibitor	broad activity (lung, breast, colon, leukemia) with IC50's of 2 to 40 pM; active against multi-drug resistance cell lines (resistant to MDR pump). NCI tumor panel, GI50's from 100 nM to 10 pM; LC50's from 100 nM to 25 pM.
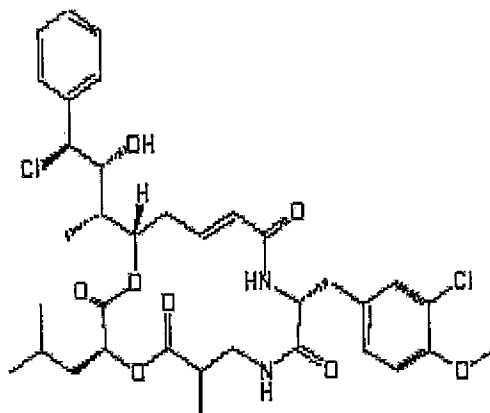
Cryptophycin 8/ semi-synthetic; starting material from Nostoc sp.	168482-36-8; 168482-40-4; 18665-94-1; 124689-65-2; 125546-14-7/ cryptophycin 5, 15 and 35	solid tumors/ not reported	tubulin polymeriza-tion inhibitor	broad spectrum anticancer activity (cell culture) including multi-drug resistant tumors

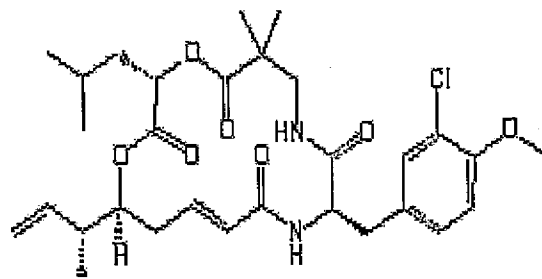|					
Cryptophycin analogs[7]/ synthetic; semi-synthetic, starting material from *Nostoc* sp./ LY-404291	219660-54-5/ LY-404292	solid tumors/ not reported		topoisomer-ase inhibitors	not reported
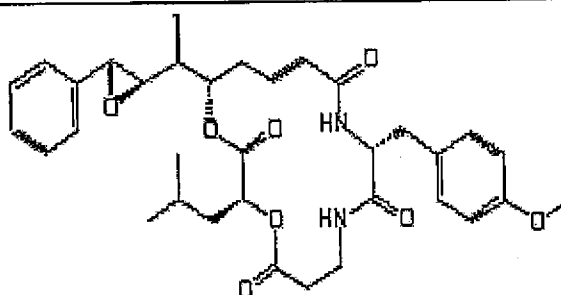
Arenastatin A analogs[8]/ *Dysidea arenaria* (marine sponge)/ Cryptophycin B; NSC-670038	not reported/ analogs prepared	cancer/ not reported		inhibits tubulin polymeriza-tion	8.7 nM (5 pg/mL) KB (nasopharyngeal); NCI tumor panel (GI50's); 100 pM to 3 pM
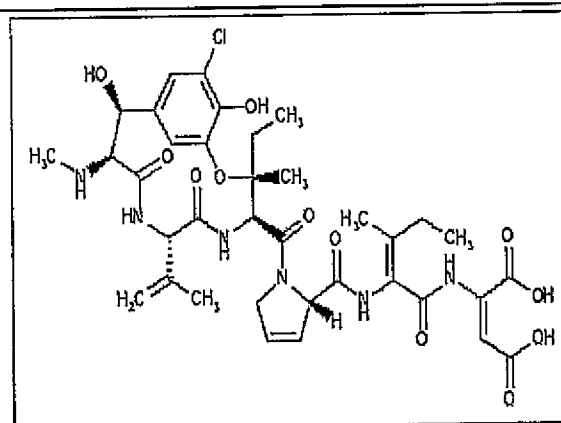
Phomopsin A/ *Diaporte toxicus* or *Phomopsin leptostromiformis* (fungi)	not reported	Liver cancer (not as potent in other cancers)/ not reported	tubulin binding agent	potent anticancer activity especially against liver cancer
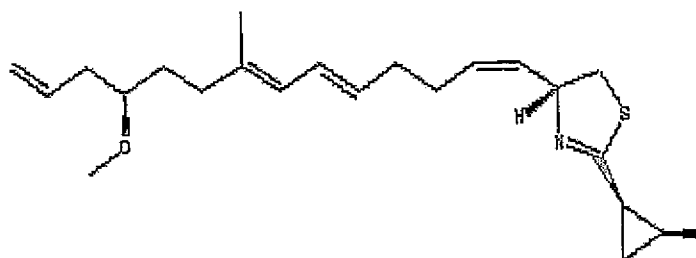
Compound/Source	CAS/Analogs	Use	Mechanism	Activity
Curacin A and analogs/ *Lyngbya majuscula* (blue green cyanobacterium)	155233-30-0/ analogs have been prepared	Cancer/ not reported	Tubulin binding agent	broad activity (cancer cell lines); 1-29 nM
Hemiasterlins A & B and analogs[9]/ *Cymbastela* sp.	not reported/ criamide A & B; geodiamiolid-G	Cancer/ not reported	Antimitotic agent (tubulin binding agent)	broad activity: 0.3-3 nM MCF7 (breast); 0.4 ng/mL P388
Spongistatins (1-9)[10]/ *Spirastrell spinispirulifera* (sea sponge)	149715-96-8; 158734-18-0; 158681-42-6; 158080-65-0; 150642-07-2; 153698-80-7; 153745-94-9; 150624-44-5; 158734-19-1/ other spongistatins	cancer/ not reported	tubulin binding agent	Most potent compounds ever tested in NCI panel cell line (mean GI50's of 0.1 nM; Spongistatin-1 GI50's of 0.025-0.035 nM with extremely potent activity against a subset of highly chemoresistant tumor types
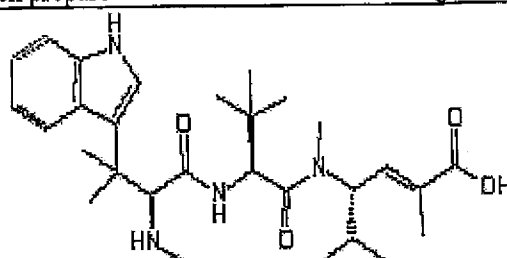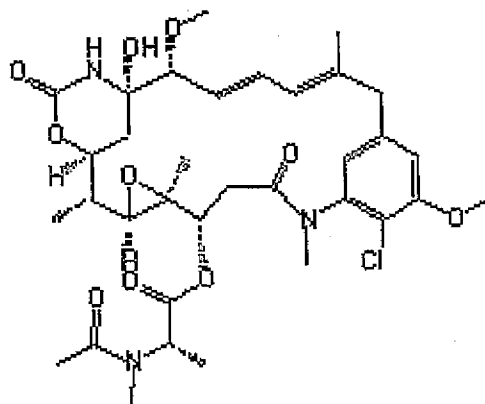
| Maytansine/ Maytenus sp./ NSC-153858 | 35846-53-8/ other related macrolides | cancer/ severe toxicity | tubulin binding agent (causes extensive disassembly of the microtubule and totally prevents tubulin spiralizaiton) | Broad Activity in NCI tumor panel (NSC-153858; NSC-153858); NCI tumor panel, GI50's from 3 μM to 0.1 pM; LC50's from 250 μM to 10 pM. Two different experiments gave very different potencies. |

| Maytansine-IgG(EGFR directed)-conjugate[11]/ semi-synthetic; starting material from Maytenus sp. | not reported/ other related macrolides | breast , head and neck, Squamous cell carcinoma/ not reported | EGFR binding and tubulin binding | not reported |
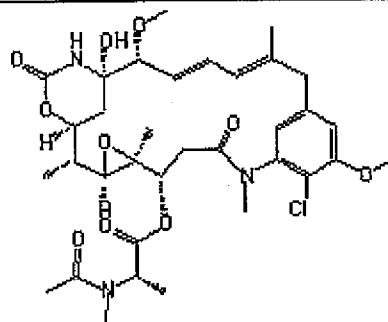
Maytansine-IgG(CD56 antigen)-conjugate[12], 3.5 drug molecules per IgG/ semi-synthetic; starting material from *Maytenus* sp./ huN901-DM1	not reported/ other related macrolides	Neuroendocrine, small-cell lung, carcinoma/ mild toxicity (fatigue, nausea, headaches and mild peripheral neuropathy); no hematological toxicity; MTD 60 mg/Kg, I.V., weekly for 4 weeks; only stable disease reported (humans)	CD56 binding and tubulin binding	antigen-specific cytotoxicity (cell culture; epidermal, breast, renal ovarian colon) with IC50's of 10-40 pM; animal studies (miceSCLC tumor--alone and in combination with taxol or cisplatin completely eliminated tumors).

Maytansine-IgG(CEA antigen)-conjugate[13], 4 drug molecules per IgG/ semi-synthetic; starting material from *Maytenus* sp./ C424-DM1	not reported/ other related macrolides	non-small-cell lung, carcinoma pancreas, lung, colon/ mild toxicity (fatigue, nausea, headaches and mild peripheral neuropathy); pancreatic lipase elevated; MTD 88 mg/Kg, I.V., every 21 days; only stable disease reported (humans); t1/2 was 44 hr.	CEA binding and tubulin binding	antigen-specific cytotoxicity (cell culture; epidermal, breast, renal ovarian colon) with IC50's of 10-40 pM; animal studies (mice: melanoma [COLO-205]--alone and in combination with taxol or cisplatin completely eliminated tumors);

Geldanamycin / *Streptomyces hygroscopicus* var. Geldanus/ NSC-212518; Antibiotic U 29135; NSC-122750	30562-34-6/ natural derivatives	cancer/ not reported	binds Hsp 90 chaperone and inhibits function	NCI tumor panel (cell culture); 5.3 to 100 nM; most active in colon, lung and leukemia. NCI tumor panel, GI50's from 10 μM to 0.1 nM; LC50's from 100 μM to 100

nM. Two assays with very different potencies.
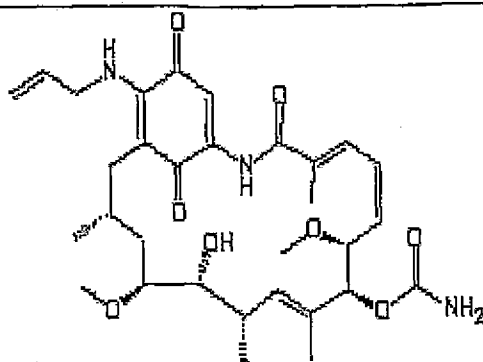
Geldanamycin Analog/ semi-synthetic; / CP-127374; 17-AAG; NSC-330507	745747-14-7/ Kosan, NCI and UK looking for analogs with longer t1/2 and oral activity; analogs include: NSC-255110; 682300; 683661; 683663.	solid tumors/ Dose limiting toxicities (anemia, anorexia, diarrhea, nausea and vomiting); t1/2 (i.v.) is about 90 min; no objective responses measured at 88 mg/Kg (i.v. daily for 5 days, every 21 days);	binds Hsp 90 chaperone and inhibits function	cell culture (not reported); animal models active (tumor regression observed) in breast, ovary, melanoma, colon.
Geldanamycin analog/ semi-synthetic; / CP-202567	not reported/ analogs prepared	solid tumors/ not reported	binds Hsp 90 chaperone and inhibits function	not reported
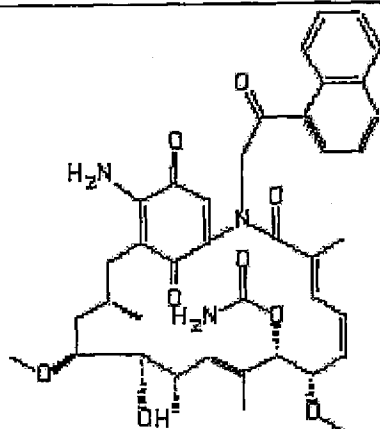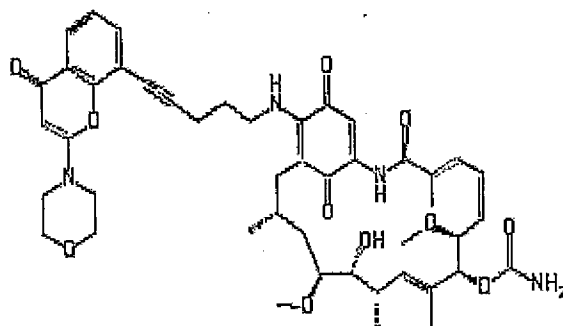
Geldanamycin conjugates/ semi-synthetic; / LY-294002-GM; PI3K-1-GM	345232-44-2/ analogs prepared	breast/ not reported	binds Hsp 90 chaperone and inhibits function; binds and inhibits PI-3 kinase	cell culture (no reported); animal models performed

Structure Not Reported

Geldanamycin Analog/ not reported/ CNF-101	not reported/ analogs prepared	breast, prostate/ not reported	binds Hsp 90 chaperone and inhibits function	not reported

Structure Not Reported

Geldanamycin-testosterone conjugate/ semi-synthetic/ GMT-1	not reported/ analogs prepared	prostate/ not reported	binds Hsp 90 chaperone and inhibits function and testosterone receptors where it is internalized	not reported; conjugate has a 15-fold selective cytotoxicity for androgen positive prostate cells
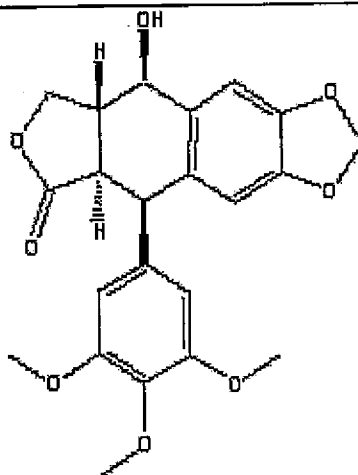
Podophyllotoxin/ *Podophyllum* sp.	518-28-5/ many analogs	Verruca vulgaris, Condyloma/ severe toxicity when	tubulin inhibitor and topoisomer-	broad activity (cell culture) with IC50's in μM range
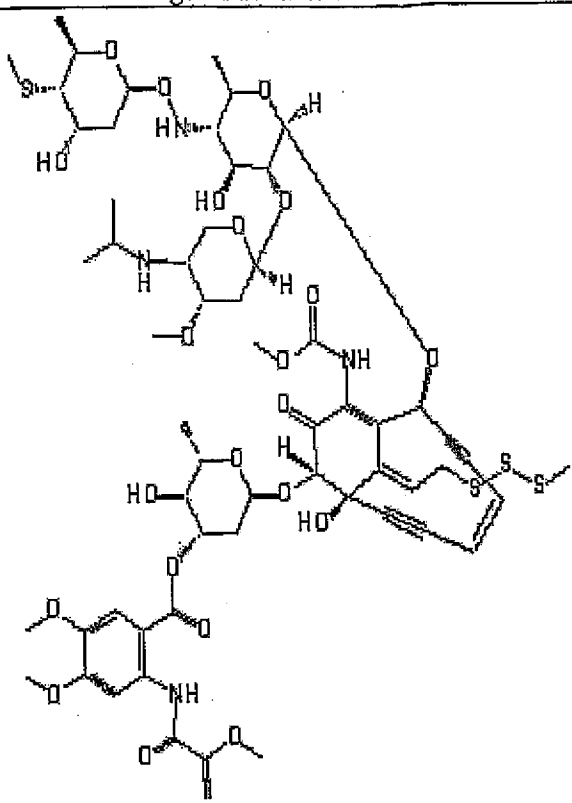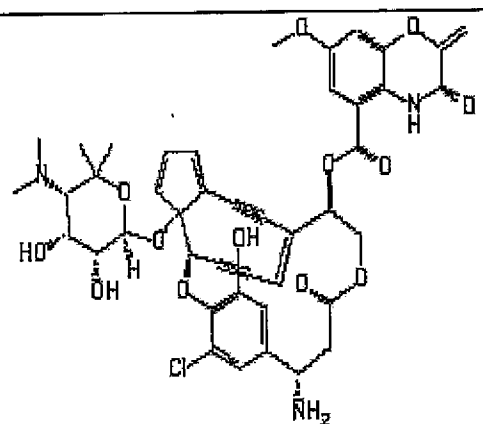
given i.v. or s.c.   ase inhibitor

esperamicin-A1/ not known/ BBM-1675A1; BMY-28175; GGM-1675	99674-26-7	cancer/ not reported (suspected severe toxicity)	DNA cleaving agent	highly potent activity (cell culture); animal models highly potent with optimal dose of 0.16 micrograms/Kg
C-1027[14]/ *Streptomyces setonii C-1027/* C-1027	120177-69-7	cancer (examined hepatoma, breast, lung and leukemia/ not reported	DNA cleaving agent	extremely potent (cell culture) IC50's in pM and fM; conjugated to antibodies the potency remains the same (ie. 5.5 to 42 pM);
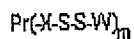
Pr(-X-S-S-W)$_m$

m = 0.5 - 15
Pr = proteinaceous carrier
W = calicheamicin minus Me-S-S-S
X = linker
Y = antibody P76.6
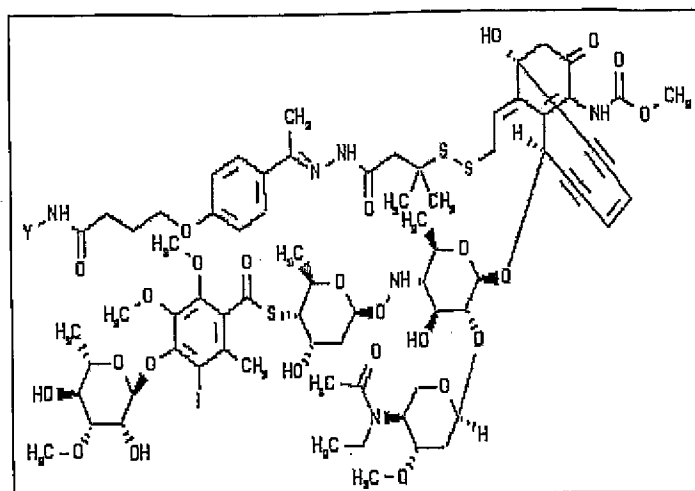
Calicheamicin-IgG(CD33 antigen)-conjugate[15]/ semi-synthetic: *Micromonospora echinospora*/ gemtuzumab ozogamicin; mylotarg; WAY-CMA-676; CMA-676; CDP-771	113440-58-7; 220578-59-6/ several reported in patents	AML/ mild toxicity	DNA cleaving agent	Kills CD33+ cells (HL-60, NOMO-1, and NKM-1) at 100 ng/mL; MDR cell lines are not effected by the drug.
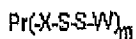
Pr(-X-S-S-W)$_m$

m = 0.5 - 15
Pr = proteinaceous carrier
W = calicheamicin minus Me-S-S-S
X = linker
Y = antibody P76.6
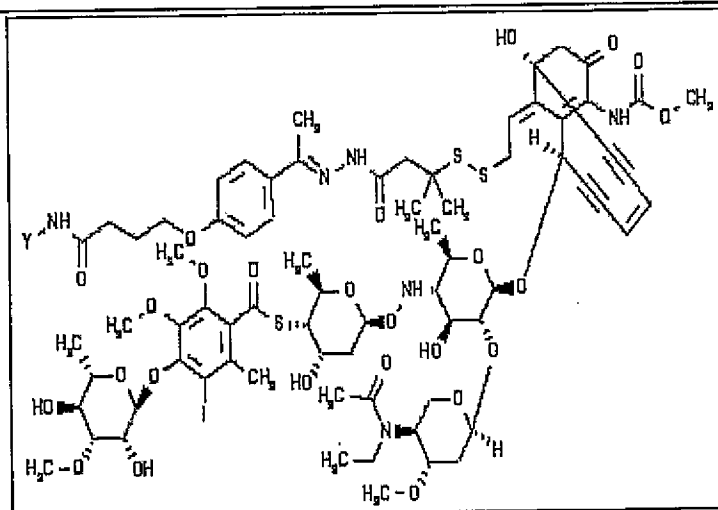
Calicheamicin-IgG-conjugates[16]/ semi-synthetic: *Micromonospora echinospora*	113440-58-7; 220578-59-6	cancer/ not reported	DNA cleaving agent	TBD
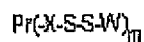
Pr(-X-S-S-W)$_m$

m = 0.5 - 15
Pr = proteinaceous carrier
W = calicheamicin minus Me-S-S-S
X = linker
Y = antibody P76.6
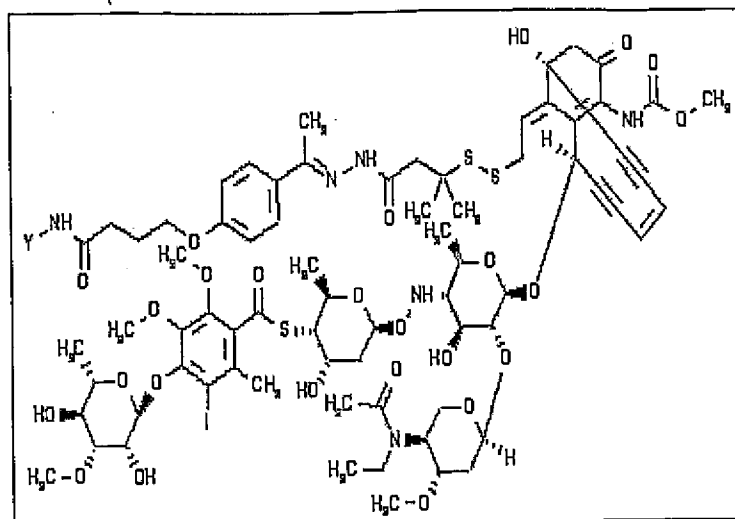
Calicheamicin-IgG(OBA1 antigen) conjugate/ semi-synthetic: *Micromonospora echinospora*/ OBA1-H8	not reported	cancer/ not reported	DNA cleaving agent	all human cancer; data not reported
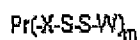
Pr(-X-S-S-W)$_m$

m = 0.5 - 15
Pr = proteinaceous carrier
W = calicheamicin minus Me-S-S-S
X = linker
Y = antibody P76.6
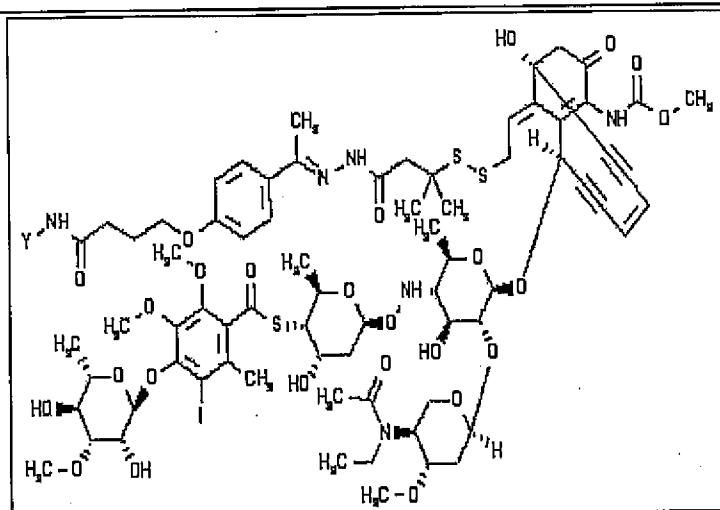
Calicheamicin-IgG(CD22 antigen) conjugate/ semi-synthetic: *Micromonospora echinospora*/ CMC-544	not reported	non-Hodgkin lymphoma, cancer/ not reported	DNA cleaving agent	all human cancer; data not reported

parially esterified polystyrene maleic acid copolymer (SMA)
conjugated to neocarzinostatin (NCS)

Neocarzinostatin[17]/ semi-synthetic; *Streptomyces*	123760-07-6; 9014-02-2	liver cancer and brain cancer/ not reported	DNA cleaving agent	cell culture data not reported.

carconistaticus/
Zinostatin stimalamer;
YM-881; YM-16881

IgG (TES-23)-conjugated to neocarzinostatin

| Neocarzinostatin/ not reported/ TES-23-NCS | not reported | solid tumors/ toxicity not reported; TES-23 antibody (without anticancer agent) was as effective at eliminating tumors as the drug conjugated protein | DNA the cleaving agent and immunostim-ulator | cell culture data not reported. |

| Kedarcidin[18]/ *Streptoalloteichus* sp NOV strain L5856, ATCC 53650/ NSC-646276 | 128512-40-3; 128512-39-0/ chromophore and protein conjugate | cancer/ not reported | DNA cleaving agent | cell culture (IC50's in ng/mL), 0.4 HCT116; 0.3 HCT116/VP35; 0.3 HCT116/VM46; 0.2 A2780; 1.3 A2780/DDP. animal models in P388 and B-16 melanoma. NCI tumor panel, GI50's from 50 μM to 5 μM. |

| Eleutherobins/ marine coral | 174545-76-7/ sarcodictyins (marine coral) | cancer/ not reported | tubulin binding agent | similar potency to taxol; not effective against MDR cell lines |

| Bryostatin-1/ *Bugula neritina* (marine bryosoan)/ GMY-45618; NSC-339555 | 83314-01-6 | leukemia, melanoma, lung, cancer/ myalgia; accumulated toxicity; poor water solubility; dose limiting toxicity | immunostim-ulant (TNF, GMCSF, etc); enhances cell kill by current anticancer agents | not reported |

| FR-901228/ *Chromobacterium violaceum* strain 968/ NSC-63-176; FK-228 | 128517-07-7 | leukemia, T-cell lymphoma, cancer/ toxic doses (LD50) 6.4 and 10 mg/Kg, ip and iv respectively; GI toxicity, lymphoid atrophy; dose limiting toxicity (human) 18 mg/Kg; t1/2 of 8 hrs | histone deacetylase inhiibitor | In vitro cell lines (NCI tumor panel); IC50's of between 0.56 and 4.1 nM (breast, lung, gastric colon, leukemia) |
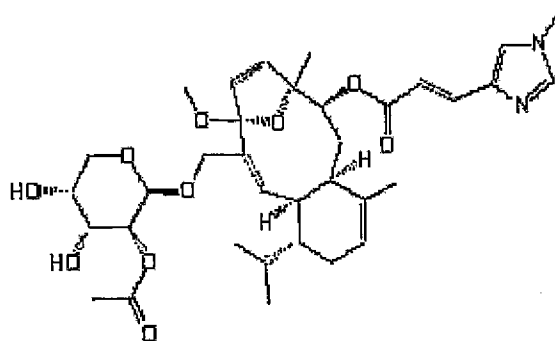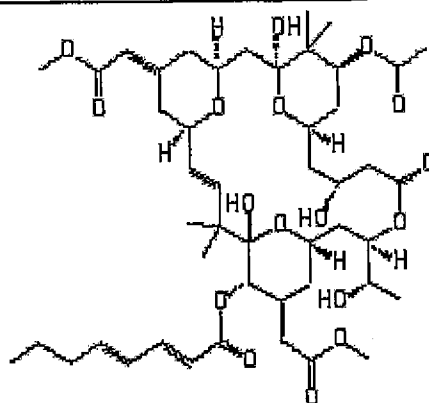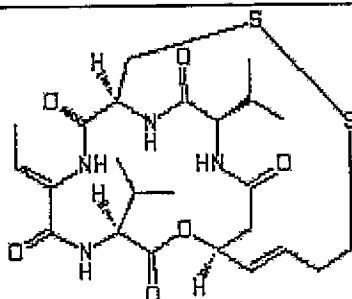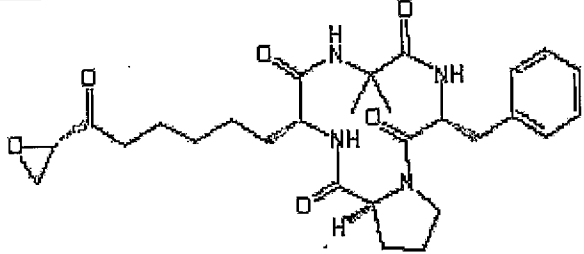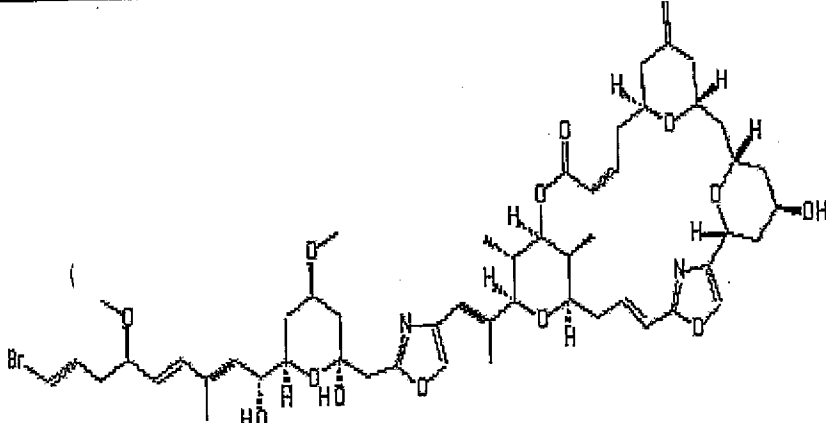
(human)

| Chlamydocin/ not reported | 53342-16-8 | cancer/ not reported | histone deacetylase inhiibitor | not reported (cell culture); inhibits histone deacetylase at an IC50 of 1.3 nM |

| Phorboxazole A[19]/ marine sponge | 181377-57-1; 165689-31-6; 180911-82-4; 165883-76-1/ analogs prepared | leukemia, myeloma/ not reported | not reported (induces apoptosis) | NCI tumor panel (details not reported); IC50's of 1-10 nM. The inhibition values (clonogenic growth of human cancer cells) at 10 nM ranged from 6.2 to > 99.9% against NALM-6 human B-lineage acute lymophoblastic leukemia cells, BT-20 breast cancer cells and U373 glioblastoma cells, with the specified compound showing inhibition values in the range of 42.4 to > 99.9% against these cell lines.; IC50's are nM for MDR cell lines. |
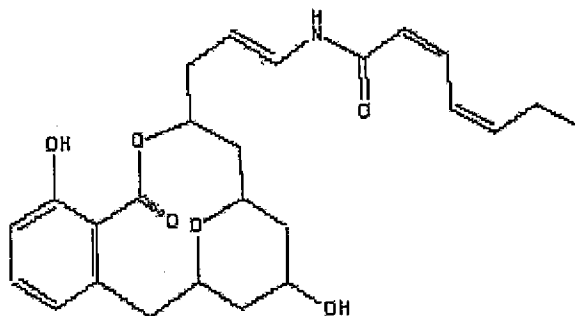
Apicularen A/ *Chondromyces robustus*	220757-06-2/ natural derivatives	cancer/ not reported	not reported	IC50's of 0.1 to 3 ng/mL (KB-3-A, KB-Va, K562, HL60, U937, A498, A549, PV3 and SK-OV3)
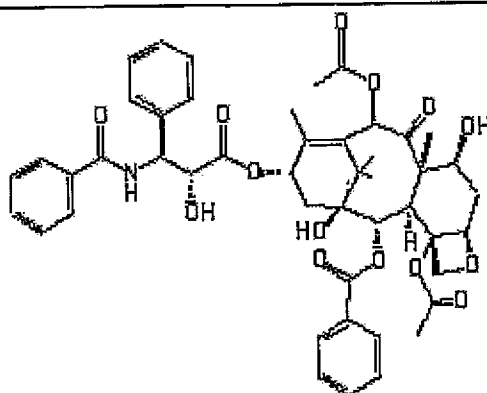
Taxol/ Pacific yew and fungi/ Paclitaxel; NSC-125973	33069624/ many analogs	cancer; breast, prostate, ovary, colon, lung, head & neck, etc./ severe toxicity (grade III and IV)	tubulin binding agent	NCI tumor panel; GI50's of 3 nM to 1 µM; TGI 50 nM to 25 µM
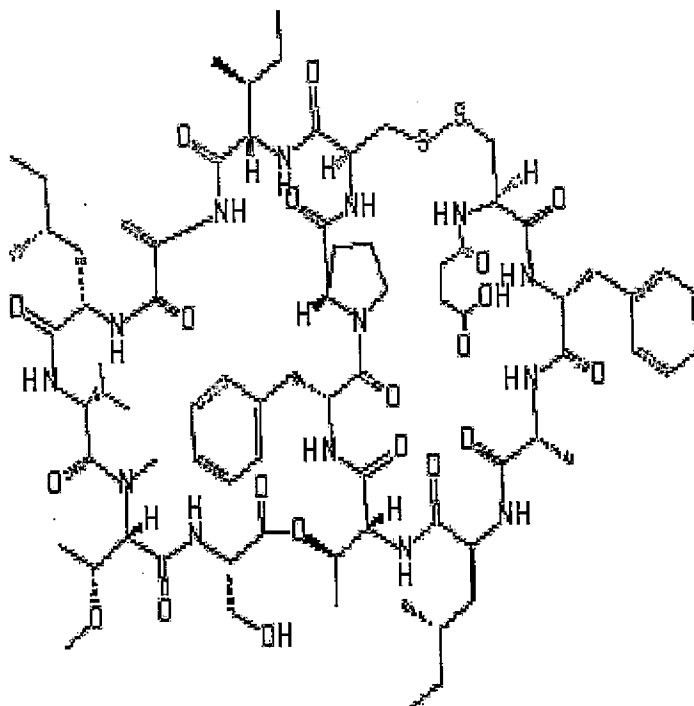
Vitilevuamide/	191681-63-7	cancer/	tubulin	cell culture; IC50's of
Didemnum cuculliferum		not reported	binding	6-311 nM (panel of
or *Polysyncraton*			agent	tumor cell lines
lithostrotum				HCT116 cells, A549
				cells, SK-MEL-5 cells
				A498 cells). The
				increase in lifespan
				(ILS) for CDF1 mice
				after ip injection of
				P388 tumor cells was in
				the range of -45 to
				+70% over the dose
				range of 0.13 to 0.006
				mg/kg.
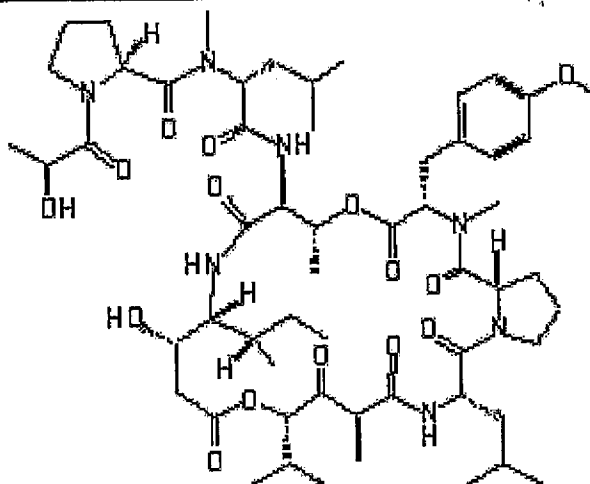
| Didemnin B/ | 77327-05-0; | non-Hodgkin's | inhibits | NCI 60-tumor panel |

Trididemnum solidum/ NSC-2325319; IND 24505	77327-04-9; 77327-06-1/ other related natural products	lymphoma, breast, carcinoma, CNS, colon/ Discontinued due to cardiotoxicity; nausea, neuro-muscular toxicity and vomiting MTD 6.3 mg/Kg; toxicity prevented achieving a clinically signif. effect; rapidly cleared (t1/2 4.8 hrs	protein synthesis via EF-1	(GI50's): 100 nM to 50 fM. Not potent against MDR cell lines.
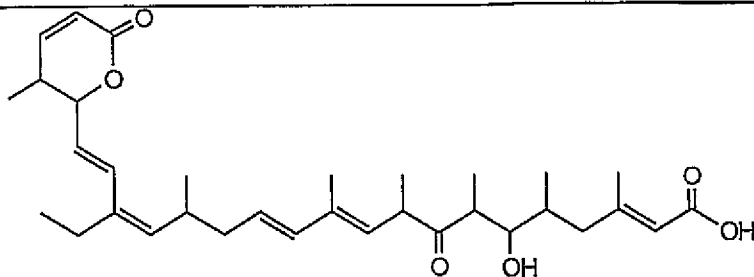
Leptomycin B/ *Streptomyces* sp. strain ATS 1287/ NSC-364372; elactocin	87081-35-4		NCI 60-tumor panel (GI50's): 8 µM to 1 pM; (LC50): 250 µM to 10 nM (several cell lines at 0.1 nM). Two testing results with very different potencies.
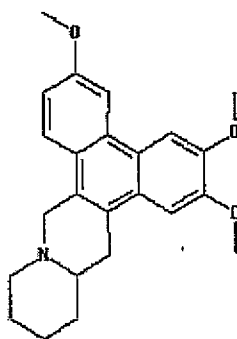
Cryptopleurin/ not known/ NSC-19912		NCI 60-tumor panel (GI50's): 19 nM to 1 pM; (LC50): 40 µM to 10 nM (several cell lines at 1 pM).
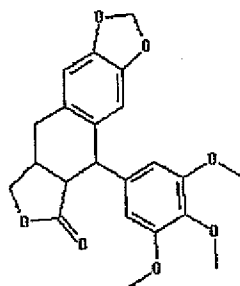
Silicicolin/
not known/
NSC-403148,
deoxypodophyllotoxin,
desoxypodophyllotoxin
podophyllotoxin,
deoxysilicicolin

19186-35-7

NCI 60-tumor panel
(GI50's): ~100 nM to 3
nM; (LC50): 50 μM to
10 nM
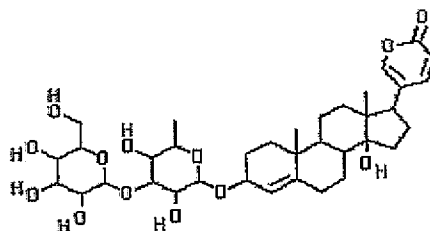
Scillaren A/
not known/
NSC-7525; Gluco-
proscillaridin A;
Scillaren A

124-99-2

NCI 60-tumor panel
(GI50's): 50 nM to 0.1
nM;
(LC50): 250 μM to 0.1
nM
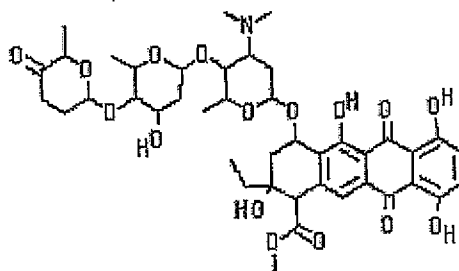
Cinerubin A-HCl/
not known/
NSC-243022; Cinerubin
A hydrochloride;
CL 86-F2 HCl;
CL-86-F2-hydrochloride

not reported

NCI 60-tumor panel
(GI50's): 15 nM to 10
pM; (LC50): 100 μM
to 6 nM

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[1] WO-09739025; US-6025466
[2] EP-00626383 30 November 1994
[3] JP-10101676
[4] WO-09705162; WO-09717364 (dolastatin synthesis and analogs)
[5] Kosan licensed patent for Epothilone analogs from Sloan-Kettering; US 00185968

[6] WO-09723211
[7] WO-09723211
[8] JP-08092232
[9] WO-09633211
[10] EP-00608111; EP-00632042; EP-00634414; WO-09748278
[11] EP-00425235; JP-53124692
[12] US-05416064; US-05208020; EP-00425235B
[13] EP-004252351 JP-53124692; US-06333410B1
[14] JP-1104183
[15] EP-00689845
[16] EP-00689845
[17] EP-00136791; EP-00087957
[18] US 50001112; US 5143906.
[19] WO-00136048

Conventional immunotoxins contain an MAb chemically conjugated to a toxin that is mutated or chemically modified to minimized binding to normal cells. Examples include anti-B4-blocked ricin, targeting CD5; and RFB4-deglycosylated ricin A chain, targeting CD22. Recombinant immunotoxins developed more recently are chimeric proteins consisting of the variable region of an antibody directed against a tumor antigen fused to a protein toxin using recombinant DNA technology. The toxin is also frequently genetically modified to remove normal tissue binding sites but retain its cytotoxicity. A large number of differentiation antigens, overexpressed receptors, or cancer-specific antigens have been identified as targets for immunotoxins, *e.g.*, CD19, CD22, CD20, IL-2 receptor (CD25), CD33, IL-4 receptor, EGF receptor and its mutants, ErB2, Lewis carbohydrate, mesothelin, transferrin receptor, GM-CSF receptor, Ras, Bcr-Abl, and c-Kit, for the treatment of a variety of malignancies including hematopoietic cancers, glioma, and breast, colon, ovarian, bladder, and gastrointestinal cancers. *See e.g.*, Brinkmann et al., *Expert Opin. Biol. Ther.* 1:693-702 (2001); Perentesis and Sievers, *Hematology/Oncology Clinics of North America* 15:677-701 (2001).

MAbs conjugated with radioisotope are used as another means of treating human malignancies, particularly hematopoietic malignancies, with a high level of specificity and effectiveness. The most commonly used isotopes for therapy are the high-energy -emitters, such as ^{131}I and ^{90}Y. Recently, ^{213}Bi-labeled anti-CD33 humanized MAb has also been tested in phase I human clinical trials. Reff et al., *supra.*

A number of MAbs have been used for therapeutic purposes. For example, the use of rituximab (Rituxan™), a recombinant chimeric anti-CD20 MAb, for treating certain

hematopoietic malignancies was approved by the FDA in 1997. Other MAbs that have since
been approved for therapeutic uses in treating human cancers include: alemtuzumab
(Campath-1H™), a humanized rat antibody against CD52; and gemtuzumab ozogamicin
(Mylotarg™), a calicheamicin-conjugated humanized mouse antCD33 MAb. The FDA is

5   also currently examining the safety and efficacy of several other MAbs for the purpose of
site-specific delivery of cytotoxic agents or radiation, e.g., radiolabeled Zevalin™ and
Bexxar™. Reff et al., supra.

A second important consideration in designing a drug delivery system is the
accessibility of a target tissue to a therapeutic agent. This is an issue of particular concern in

10 the case of treating a disease of the central nervous system (CNS), where the blood-brain ·
barrier prevents the diffusion of macromolecules. Several approaches have been developed
to bypass the blood-brain barrier for effective delivery of therapeutic agents to the CNS.

The understanding of iron transport mechanism from plasma to brain provides a
useful tool in bypassing the blood-brain barrier (BBB). Iron, transported in plasma by

15 transferrin, is an essential component of virtually all types of cells. The brain needs iron for
metabolic processes and receives iron through transferrin receptors located on brain capillary
endothelial cells via receptor-mediated transcytosis and endocytosis. Moos and Morgan,
Cellular and Molecular Neurobiology 20:77-95 (2000). Delivery systems based on
transferrin-transferrin receptor interaction have been established for the efficient delivery of

20 peptides, proteins, and liposomes into the brain. For example, peptides can be coupled with a
Mab directed against the transferrin receptor to achieve greater uptake by the brain, Moos and
Morgan, Supra. Similarly, when coupled with an MAb directed against the transferrin
receptor, the transportation of basic fibroblast growth factor (bFGF) across the blood-brain
barrier is enhanced. Song et al., The Journal of Pharmacology and Experimental

25 Therapeutics 301:605-610 (2002); Wu et al., Journal of Drug Targeting 10:239-245 (2002).
In addition, a liposomal delivery system for effective transport of the chemotherapy drug,
doxorubicin, into C6 glioma has been reported, where transferrin was attached to the distal
ends of liposomal PEG chains. Eavarone et al., J. Biomed. Mater. Res. 51:10-14 (2000). A
number of US patents also relate to delivery methods bypassing the blood-brain barrier based

30 on transferrin-transferrin receptor interaction. See e.g., US Patent Nos. 5,154,924;
5,182,107; 5,527,527; 5,833,988; 6,015,555.

There are other suitable conjugation partners for a pharmaceutical agent to bypass the blood-brain barrier. For example, US Patent Nos. 5,672,683, 5,977,307 and WO 95/02421 relate to a method of delivering a neuropharmaceutical agent across the blood-brain barrier, where the agent is administered in the form of a fusion protein with a ligand that is reactive

5   with a brain capillary endothelial cell receptor; WO 99/00150 describes a drug delivery system in which the transportation of a drug across the blood-brain barrier is facilitated by conjugation with an MAb directed against human insulin receptor; WO 89/10134 describes a chimeric peptide, which includes a peptide capable of crossing the blood brain barrier at a relatively high rate and a hydrophilic neuropeptide incapable of transcytosis, as a means of

10   introducing hydrophilic neuropeptides into the brain; WO 01/60411 A1 provides a pharmaceutical composition that can easily transport a pharmaceutically active ingredient into the brain. The active ingredient is bound to a hibernation-specific protein that is used as a conjugate, and administered with a thyroid hormone or a substance promoting thyroid hormone production. In addition, an alternative route of drug delivery for bypassing the

15   blood-brain barrier has been explored. For instance, intranasal delivery of therapeutic agents without the need for conjugation has been shown to be a promising alternative delivery method (Frey, 2002, Drug Delivery Technology, 2(5):46-49).

In addition to facilitating the transportation of drugs across the blood-brain barrier, transferrin-transferrin receptor interaction is also useful for specific targeting of certain tumor

20   cells, as many tumor cells overexpress transferrin receptor on their surface. This strategy has been used for delivering bioactive macromolecules into K562 cells via a transferrin conjugate (Wellhoner et al., *The Journal of Biological Chemistry* 266:4309-4314 (1991)), and for delivering insulin into enterocyte-like Caco-2 cells via a transferrin conjugate (Shah and Shen, *Journal of Pharmaceutical Sciences* 85:1306-1311 (1996)).

25   Furthermore, as more becomes known about the functions of various iron transport proteins, such as lactotransferrin receptor, melanotransferrin, ceruloplasmin, and Divalent Cation Transporter and their expression pattern, some of the proteins involved in iron transport mechanism(e.g., melanotransferrin), or their fragments, have been found to be similarly effective in assisting therapeutic agents transport across the blood-brain barrier or

30   targeting specific tissues (WO 02/13843 A2, WO 02/13873 A2). For a review on the use of

transferrin and related proteins involved in iron uptake as conjugates in drug delivery, *see* Li
and Qian, *Medical Research Reviews* 22:225-250 (2002).

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The concept of tissue-specific delivery of therapeutic agents goes beyond the
interaction between transferrin and transferrin receptor or their related proteins. For example,
a bone-specific delivery system has been described in which proteins are conjugated with a
bone-seeking aminobisphosphate for improved delivery of proteins to mineralized tissue.
Uludag and Yang, *Biotechnol. Prog.* 18:604-611 (2002). For a review on this topic, *see* Vyas
et al., *Critical Reviews in Therapeutic Drug Carrier System* 18:1-76 (2001).

A variety of linkers may be used in the process of generating bioconjugates for the
purpose of specific delivery of therapeutic agents,. Suitable linkers include homo- and
heterobifunctional cross-linking reagents, which may be cleavable by, *e.g.*, acid-catalyzed
dissociation, or non-cleavable (*see, e.g.*, Srinivasachar and Neville, *Biochemistry* 28:2501-
2509 (1989); Wellhoner et al., *The Journal of Biological Chemistry* 266:4309-4314 (1991)).
Interaction between many known binding partners, such as biotin and avidin/streptavidin, can
also be used as a means to join a therapeutic agent and a conjugate partner that ensures the
specific and effective delivery of the therapeutic agent. Using the methods of the invention,
proteins may be used to deliver molecules to intracellular compartments as conjugates.
Proteins, peptides, hormones, cytokines, small molecules or the like that bind to specific cell
surface receptors that are internalized after ligand binding may be used for intracellular
targeting of conjugated therapeutic compounds. Typically, the receptor-ligand complex is
internalized into intracellular vesicles that are delivered to specific cell compartments,
including, but not limited to, the nucleus, mitochondria, golgi, ER, lysosome, and endosome,
depending on the intracellular location targeted by the receptor. By conjugating the receptor
ligand with the desired molecule, the drug will be carried with the receptor-ligand complex
and be delivered to the intracellular compartments normally targeted by the receptor. The
drug can therefore be delivered to a specific intracellular location in the cell where it is
needed to treat a disease.

Many proteins may be used to target therapeutic agents to specific tissues and organs.
Targeting proteins include, but are not limited to, growth factors (EPO, HGH, EGF, nerve
growth factor, FGF, among others), cytokines (GM-CSF, G-CSF, the interferon family,
interleukins, among others), hormones (FSH, LH, the steroid families, estrogen,

corticosteroids, insulin, among others), serum proteins (albumin, lipoproteins, fetoprotein, human serum proteins, antibodies and fragments of antibodies, among others), and vitamins (folate, vitamin C, vitamin A, among others). Targeting agents are available that are specific for receptors on most cells types.

5   Contemplated linkage configurations include, but are not limited to, protein-sugar-linker-sugar-protein and multivalent forms thereof, protein-sugar-linker-protein and multivalent forms thereof, protein-sugar-linker-therapeutic agent, where the therapeutic agent includes, but are not limited to, small molecules, peptides and lipids. In some embodiments, a hydrolysable linker is used that can be hydrolyzed once internalized. An acid labile linker

10   can be used to advantage where the protein conjugate is internalized into the endosomes or lysosomes which have an acidic pH. Once internalized into the endosome or lysosome, the linker is hydrolyzed and the therapeutic agent is released from the targeting agent.

          In an exemplary embodiment, transferrin is conjugated via a linker to an enzyme or a nucleic acid vector that encoded the enzyme desired to be targeted to a cell that presents

15   transferrin receptors in a patient. The patient could, for example, require enzyme replacement therapy for that particular enzyme. In particularly preferred embodiments, the enzyme is one that is lacking in a patient with a lysosomal storage disease (see Table 5). Once in circulation, the transferrin-enzyme conjugate is linked to transferrin receptors and is internalized in early endosomes (Xing et al., 1998, Biochem. J. 336:667; Li et al., 2002,

20   Trends in Pharmcol. Sci. 23:206; Suhaila et al., 1998, J. Biol. Chem. 273:14355). Other contemplated targeting agents that are related to transferrin include, but are not limited to, lactotransferrin (lactoferrin), melanotransferrin (p97), ceruloplasmin, and divalent cation transporter.

          In another exemplary embodiment, transferrin-dystrophin conjugates would enter

25   endosomes by the transferrin pathway. Once there, the dystrophin is released due to a hydrolysable linker which can then be taken to the intracellular compartment where it is required. This embodiment may be used to treat a patient with muscular dystrophy by supplementing a genetically defective dystrophin gene and/or protein with the functional dystrophin peptide connected to the transferrin.

30

E. Therapeutic Moieties

In another preferred embodiment, the modified sugar includes a therapeutic moiety. Those of skill in the art will appreciate that there is overlap between the category of therapeutic moieties and biomolecules; many biomolecules have therapeutic properties or

5   potential.

The therapeutic moieties can be agents already accepted for clinical use or they can be drugs whose use is experimental, or whose activity or mechanism of action is under investigation. The therapeutic moieties can have a proven action in a given disease state or can be only hypothesized to show desirable action in a given disease state. In a preferred

10   embodiment, the therapeutic moieties are compounds, which are being screened for their ability to interact with a tissue of choice. Therapeutic moieties, which are useful in practicing the instant invention include drugs from a broad range of drug classes having a variety of pharmacological activities. In some embodiments, it is preferred to use therapeutic moieties that are not sugars. An exception to this preference is the use of a sugar that is modified by

15   covalent attachment of another entity, such as a PEG, biomolecule, therapeutic moiety, diagnostic moiety and the like. In an exemplary embodiment, an antisense nucleic acid moeity is conjugated to a linker arm which is attached to the targeting moiety. In another exemplary embodiment, a therapeutic sugar moiety is conjugated to a linker arm and the sugar-linker arm cassette is subsequently conjugated to a peptide via a method of the

20   invention.

Methods of conjugating therapeutic and diagnostic agents to various other species are well known to those of skill in the art. *See,* for example Hermanson, BIOCONJUGATE TECHNIQUES, Academic Press, San Diego, 1996; and Dunn *et al.,* Eds. POLYMERIC DRUGS AND DRUG DELIVERY SYSTEMS, ACS Symposium Series Vol. 469, American Chemical

25   Society, Washington, D.C. 1991.

In an exemplary embodiment, the therapeutic moiety is attached to the modified sugar via a linkage that is cleaved under selected conditions. Exemplary conditions include, but are not limited to, a selected pH (e.g., stomach, intestine, endocytotic vacuole), the presence of an active enzyme (e.g., esterase, protease, reductase, oxidase), light, heat and the like. Many

30   cleavable groups are known in the art. *See,* for example, Jung *et al., Biochem. Biophys. Acta,* **761**: 152-162 (1983); Joshi *et al., J. Biol. Chem.,* **265**: 14518-14525 (1990); Zarling *et al., J.*

Immunol., **124**: 913-920 (1980); Bouizar *et al.*, *Eur. J. Biochem.*, **155**: 141-147 (1986); Park *et al.*, *J. Biol. Chem.*, **261**: 205-210 (1986); Browning *et al.*, *J. Immunol.*, **143**: 1859-1867 (1989).

5   Classes of useful therapeutic moieties include, for example, non-steroidal anti-inflammatory drugs (NSAIDS). The NSAIDS can, for example, be selected from the following categories: (*e.g.*, propionic acid derivatives, acetic acid derivatives, fenamic acid derivatives, biphenylcarboxylic acid derivatives and oxicams); steroidal anti-inflammatory drugs including hydrocortisone and the like; adjuvants; antihistaminic drugs (*e.g.*, chlorpheniramine, triprolidine); antitussive drugs (*e.g.*, dextromethorphan, codeine,

10   caramiphen and carbetapentane); antipruritic drugs (*e.g.*, methdilazine and trimeprazine); anticholinergic drugs (*e.g.*, scopolamine, atropine, homatropine, levodopa); anti-emetic and antinauseant drugs (*e.g.*, cyclizine, meclizine, chlorpromazine, buclizine); anorexic drugs (*e.g.*, benzphetamine, phentermine, chlorphentermine, fenfluramine); central stimulant drugs (*e.g.*, amphetamine, methamphetamine, dextroamphetamine and methylphenidate);

15   antiarrhythmic drugs (*e.g.*, propanolol, procainamide, disopyramide, quinidine, encainide); β-adrenergic blocker drugs (*e.g.*, metoprolol, acebutolol, betaxolol, labetalol and timolol); cardiotonic drugs (*e.g.*, milrinone, amrinone and dobutamine); antihypertensive drugs (*e.g.*, enalapril, clonidine, hydralazine, minoxidil, guanadrel, guanethidine);diuretic drugs (*e.g.*, amiloride and hydrochlorothiazide); vasodilator drugs (*e.g.*, diltiazem, amiodarone,

20   isoxsuprine, nylidrin, tolazoline and verapamil); vasoconstrictor drugs (*e.g.*, dihydroergotamine, ergotamine and methylsergide); antiulcer drugs (*e.g.*, ranitidine and cimetidine); anesthetic drugs (*e.g.*, lidocaine, bupivacaine, chloroprocaine, dibucaine); antidepressant drugs (*e.g.*, imipramine, desipramine, amitryptiline, nortryptiline); tranquilizer and sedative drugs (*e.g.*, chlordiazepoxide, benacytyzine, benzquinamide, flurazepam,

25   hydroxyzine, loxapine and promazine); antipsychotic drugs (*e.g.*, chlorprothixene, fluphenazine, haloperidol, molindone, thioridazine and trifluoperazine); antimicrobial drugs (antibacterial, antifungal, antiprotozoal and antiviral drugs).

        Classes of useful therapeutic moieties include adjuvants. The adjuvants can, for example, be selected from keyhole lymphet hemocyanin conjugates, monophosphoryl lipid

30   A, mycoplasma-derived lipopeptide MALP-2, cholera toxin B subunit, *Escherichia coli* heat-labile toxin, universal T helper epitope from tetanus toxoid, interleukin-12, CpG

oligodeoxynucleotides, dimethyldioctadecylammonium bromide, cyclodextrin, squalene, aluminum salts, meningococcal outer membrane vesicle (OMV), montanide ISA, TiterMax™ (available from Sigma, St. Louis MO), nitrocellulose absorption, immune-stimulating complexes such as Quil A, Gerbu™ adjuvant (Gerbu Biotechnik, Kirchwald, Germany),

5   threonyl muramyl dipeptide, thymosin alpha, bupivacaine, GM-CSF, Incomplete Freund's Adjuvant, MTP-PE/MF59 (Ciba/Geigy, Basel, Switzerland), polyphosphazene, saponin derived from the soapbark tree *Quillaja saponaria*, and Syntex adjuvant formulation (Biocine, Emeryville, CA), among others well known to those in the art.

Antimicrobial drugs which are preferred for incorporation into the present

10   composition include, for example, pharmaceutically acceptable salts of β-lactam drugs, quinolone drugs, ciprofloxacin, norfloxacin, tetracycline, erythromycin, amikacin, triclosan, doxycycline, capreomycin, chlorhexidine, chlortetracycline, oxytetracycline, clindamycin, ethambutol, hexamidine isothionate, metronidazole, pentamidine, gentamycin, kanamycin, lineomycin, methacycline, methenamine, minocycline, neomycin, netilmycin, paromomycin,

15   streptomycin, tobramycin, miconazole and amantadine.

Other drug moieties of use in practicing the present invention include antineoplastic drugs (*e.g.*, antiandrogens (*e.g.*, leuprolide or flutamide), cytocidal agents (*e.g.*, adriamycin, doxorubicin, taxol, cyclophosphamide, busulfan, cisplatin, β-2-interferon) anti-estrogens (*e.g.*, tamoxifen), antimetabolites (*e.g.*, fluorouracil, methotrexate, mercaptopurine,

20   thioguanine). Also included within this class are radioisotope-based agents for both diagnosis and therapy, and conjugated toxins, such as ricin, geldanamycin, mytansin, CC-1065, C-1027, the duocarmycins, calicheamycin and related structures and analogues thereof, and the toxins listed in Table 2.

The therapeutic moiety can also be a hormone (*e.g.*, medroxyprogesterone, estradiol,

25   leuprolide, megestrol, octreotide or somatostatin); muscle relaxant drugs (*e.g.*, cinnamedrine, cyclobenzaprine, flavoxate, orphenadrine, papaverine, mebeverine, idaverine, ritodrine, diphenoxylate, dantrolene and azumolen); antispasmodic drugs; bone-active drugs (*e.g.*, diphosphonate and phosphonoalkylphosphinate drug compounds); endocrine modulating drugs (*e.g.*, contraceptives (*e.g.*, ethinodiol, ethinyl estradiol, norethindrone, mestranol,

30   desogestrel, medroxyprogesterone), modulators of diabetes (*e.g.*, glyburide or chlorpropamide), anabolics, such as testolactone or stanozolol, androgens (*e.g.*,

methyltestosterone, testosterone or fluoxymesterone), antidiuretics (*e.g.*, desmopressin) and calcitonins).

Also of use in the present invention are estrogens (*e.g.*, diethylstilbesterol), glucocorticoids (*e.g.*, triamcinolone, betamethasone, etc.) andprogesterones, such as

5   norethindrone, ethynodiol, norethindrone, levonorgestrel; thyroid agents (*e.g.*, liothyronine or levothyroxine) or anti-thyroid agents (*e.g.*, methimazole); antihyperprolactinemic drugs (*e.g.*, cabergoline); hormone suppressors (*e.g.*, danazol or goserelin), oxytocics (*e.g.*, methylergonovine or oxytocin) and prostaglandins, such as mioprostol, alprostadil or dinoprostone, can also be employed.

10   Other useful modifying groups include immunomodulating drugs (*e.g.*, antihistamines, mast cell stabilizers, such as lodoxamide and/or cromolyn, steroids (*e.g.*, triamcinolone, beclomethazone, cortisone, dexamethasone, prednisolone, methylprednisolone, beclomethasone, or clobetasol), histamine H2 antagonists (*e.g.*, famotidine, cimetidine, ranitidine), immunosuppressants (*e.g.*, azathioprine, cyclosporin), etc.

15 Groups with anti-inflammatory activity, such as sulindac, etodolac, ketoprofen and ketorolac, are also of use. Other drugs of use in conjunction with the present invention will be apparent to those of skill in the art.

Classes of useful therapeutic moieties include, for example, antisense drugs and also naked DNA. The antisense drugs can be selected from for example Affinitak (ISIS,

20 Carlsbad, CA) and Genasense ™ (from Genta, Berkeley Heights, NJ). Naked DNA can be delivered as a gene therapy therapeutic for example with the DNA encoding for example factors VIII and IX for treatment of hemophilia disorders.

F. Preparation of Modified Sugars

25   Modified sugars useful in forming the conjugates of the invention are discussed herein. The discussion focuses on preparing a sugar modified with a water-soluble polymer for clarity of illustration. In particular, the discussion focuses on the preparation of modified sugars that include a poly(ethylene glycol) moiety. Those of skill will appreciate that the methods set forth herein are broadly applicable to the preparation of modified sugars,

30 therefore, the discussion should not be interpreted as limiting the scope of the invention.

In general, the sugar moiety and the modifying group are linked together through the use of reactive groups, which are typically transformed by the linking process into a new organic functional group or unreactive species. The sugar reactive functional group(s), is located at any position on the sugar moiety. Reactive groups and classes of reactions useful

5   in practicing the present invention are generally those that are well known in the art of bioconjugate chemistry. Currently favored classes of reactions available with reactive sugar moieties are those, which proceed under relatively mild conditions. These include, but are not limited to nucleophilic substitutions (*e.g.*, reactions of amines and alcohols with acyl halides, active esters), electrophilic substitutions (*e.g.*, enamine reactions) and additions to

10   carbon-carbon and carbon-heteroatom multiple bonds (*e.g.*, Michael reaction, Diels-Alder addition). These and other useful reactions are discussed in, for example, Smith and March, ADVANCED ORGANIC CHEMISTRY, 5th Ed., John Wiley & Sons, New York, 2001; Hermanson, BIOCONJUGATE TECHNIQUES, Academic Press, San Diego, 1996; and Feeney *et al.*, MODIFICATION OF PROTEINS; Advances in Chemistry Series, Vol. 198, American

15   Chemical Society, Washington, D.C., 1982.

Useful reactive functional groups pendent from a sugar nucleus or modifying group include, but are not limited to:

(a) carboxyl groups and various derivatives thereof including, but not limited to, N-hydroxysuccinimide esters, N-hydroxybenzotriazole esters, acid halides, acyl imidazoles,

20   thioesters, p-nitrophenyl esters, alkyl, alkenyl, alkynyl and aromatic esters;

(b) hydroxyl groups, which can be converted to, *e.g.*, esters, ethers, aldehydes, *etc.*

(c) haloalkyl groups, wherein the halide can be later displaced with a nucleophilic group such as, for example, an amine, a carboxylate anion, thiol anion, carbanion, or an alkoxide ion, thereby resulting in the covalent attachment of a new group at the functional

25   group of the halogen atom;

(d) dienophile groups, which are capable of participating in Diels-Alder reactions such as, for example, maleimido groups;

(e) aldehyde or ketone groups, such that subsequent derivatization is possible via formation of carbonyl derivatives such as, for example, imines, hydrazones, semicarbazones

30   or oximes, or via such mechanisms as Grignard addition or alkyllithium addition;

(f) sulfonyl halide groups for subsequent reaction with amines, for example, to form sulfonamides;

(g) thiol groups, which can be, for example, converted to disulfides or reacted with alkyl and acyl halides;

5   (h) amine or sulfhydryl groups, which can be, for example, acylated, alkylated or oxidized;

(i) alkenes, which can undergo, for example, cycloadditions, acylation, Michael addition, *etc*; and

(j) epoxides, which can react with, for example, amines and hydroxyl compounds.

10   The reactive functional groups can be chosen such that they do not participate in, or interfere with, the reactions necessary to assemble the reactive sugar nucleus or modifying group. Alternatively, a reactive functional group can be protected from participating in the reaction by the presence of a protecting group. Those of skill in the art understand how to protect a particular functional group such that it does not interfere with a chosen set of

15   reaction conditions. For examples of useful protecting groups, *see*, for example, Greene *et al.*, PROTECTIVE GROUPS IN ORGANIC SYNTHESIS, John Wiley & Sons, New York, 1991.

In the discussion that follows, a number of specific examples of modified sugars that are useful in practicing the present invention are set forth. In the exemplary embodiments, a sialic acid derivative is utilized as the sugar nucleus to which the modifying group is

20   attached. The focus of the discussion on sialic acid derivatives is for clarity of illustration only and should not be construed to limit the scope of the invention. Those of skill in the art will appreciate that a variety of other sugar moieties can be activated and derivatized in a manner analogous to that set forth using sialic acid as an example. For example, numerous methods are available for modifying galactose, glucose, N-acetylgalactosamine and fucose to

25   name a few sugar substrates, which are readily modified by art recognized methods. *See*, for example, Elhalabi *et al.*, *Curr. Med. Chem.* 6: 93 (1999); and Schafer *et al.*, *J. Org. Chem.* 65: 24 (2000).

In an exemplary embodiment, the peptide that is modified by a method of the invention is a peptide that is produced in mammalian cells (e.g., CHO cells) or in a transgenic

30   animal and thus, contains N- and/or O-linked oligosaccharide chains, which are incompletely sialylated. The oligosaccharide chains of the glycopeptide lacking a sialic acid and

containing a terminal galactose residue can be PEGylated, PPGylated or otherwise modified with a modified sialic acid.

In Scheme 4, the mannosamine glycoside 1, is treated with the active ester of a protected amino acid (*e.g.*, glycine) derivative, converting the sugar amine residue into the corresponding protected amino acid amide adduct. The adduct is treated with an aldolase to form the sialic acid 2. Compound 2 is converted to the corresponding CMP derivative by the action of CMP-SA synthetase, followed by catalytic hydrogenation of the CMP derivative to produce compound 3. The amine introduced via formation of the glycine adduct is utilized as a locus of PEG or PPG attachment by reacting compound 3 with an activated PEG or PPG derivative (*e.g.*, PEG-C(O)NHS, PPG-C(O)NHS), producing 4 or 5, respectively.

Scheme 4

CMP-SA-5-NHCOCH2NH—**PEG**

CMP-SA-5-NHCOCH2NH—**PPG**

5
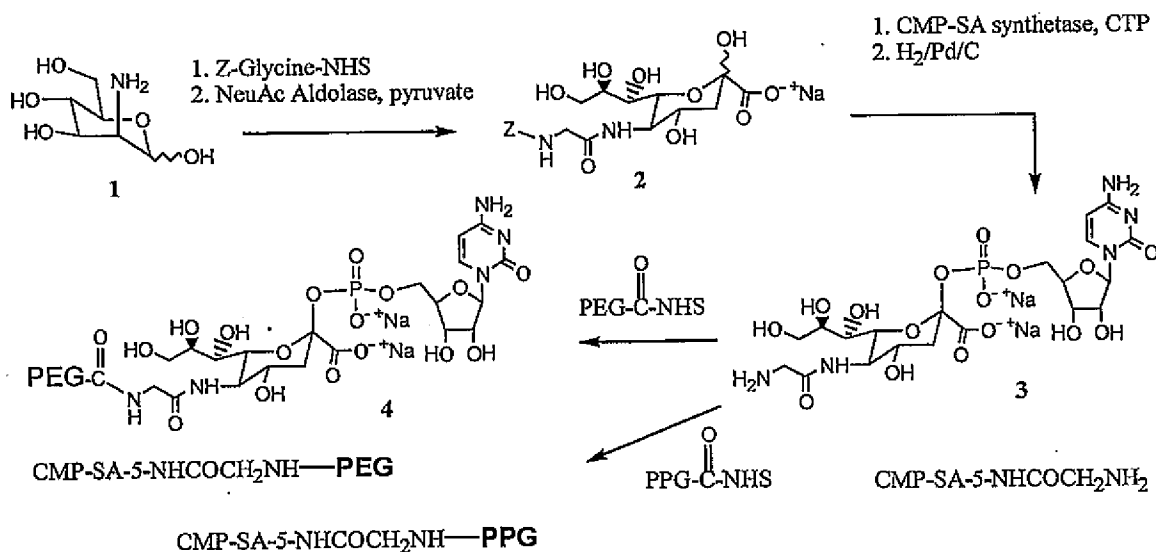
Table 3 sets forth representative examples of sugar monophosphates that are derivatized with a PEG or PPG moiety. Certain of the compounds of Table 3 are prepared by the method of Scheme 1. Other derivatives are prepared by art-recognized methods. *See*, for example, Keppler *et al.*, *Glycobiology* **11**: 11R (2001); and Charter *et al.*, *Glycobiology* **10**: 1049 (2000)). Other amine reactive PEG and PPG analogues are commercially available, or they can be prepared by methods readily accessible to those of skill in the art.

Table 3. Examples of sugar monophosphates that are derivatized with a PEG or PPG moiety

CMP-KDN-5-O-R

CMP-NeuAc-9-NH-R

CMP-NeuAc-8-O-R

CMP-NeuAc-8-NH-R

CMP-NeuAc-7-O-R

CMP-NeuAc-7-NH-R

CMP-NeuAc-4-O-R

CMP-NeuAc-4-NH-R

-162-
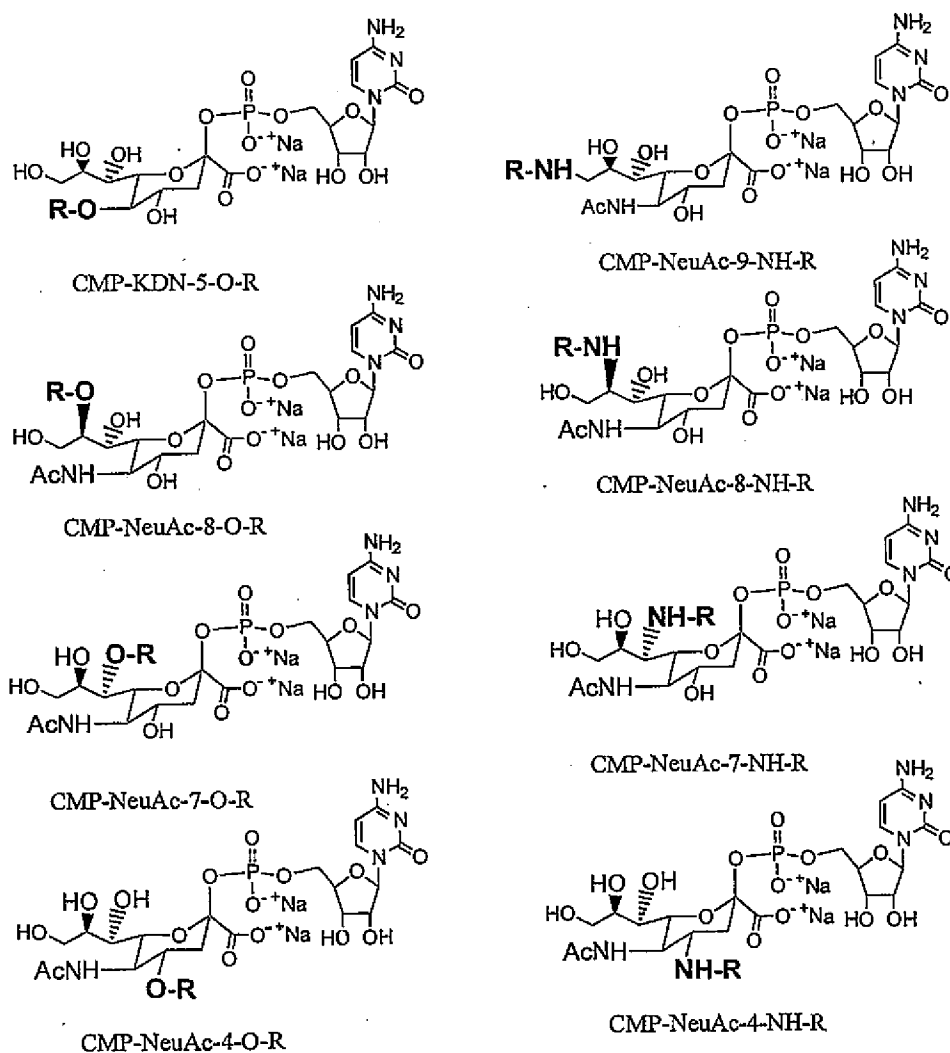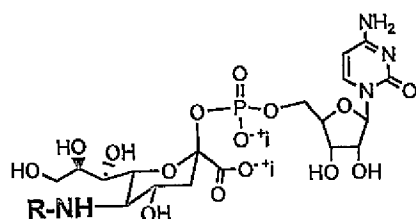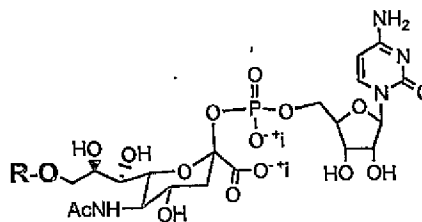
CMP-SA-5-NH-R   CMP-NeuAc-9-O-R

The modified sugar phosphates of use in practicing the present invention can be
substituted in other positions as well as those set forth above. "i" may be Na or another salt
and "i" may be interchangeable with Na. Presently preferred substitutions of sialic acid are
set forth in Formula 5.

Formula 5:
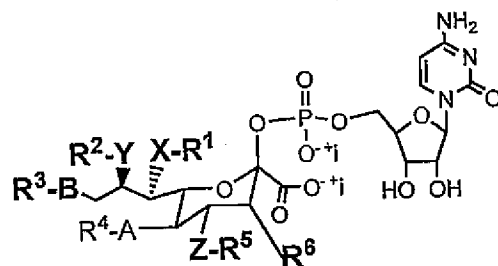
(I)

in which X is a linking group, which is preferably selected from –O-, -N(H)-, -S,
CH_2-, and $N(R)_2$, in which each R is a member independently selected from R^1-R^5. "i" may
be Na or another salt, and Na may be interchangeable with "i:The symbols Y, Z, A and B
each represent a group that is selected from the group set forth above for the identity of X. X,
Y, Z, A and B are each independently selected and, therefore, they can be the same or
different. The symbols R^1, R^2, R^3, R^4 and R^5 represent H, polymers, a water-soluble polymer,
therapeutic moiety, biomolecule or other moiety. The symbol R6 represents H, OH, or a
polymer. Alternatively, these symbols represent a linker that is linked to a polymer, water-
soluble polymer, therapeutic moiety, biomolecule or other moiety.

In another exemplary embodiment, a mannosamine is simultaneously acylated and
activated for a nucleophilic substitution by the use of chloroacetic anhydride as set forth in
Scheme 5. In each of the schemes presented in this section, i^+ or Na^+ can be interchangeable,
wherein the salt can be sodium, or can be any other suitable salt.

Scheme 5
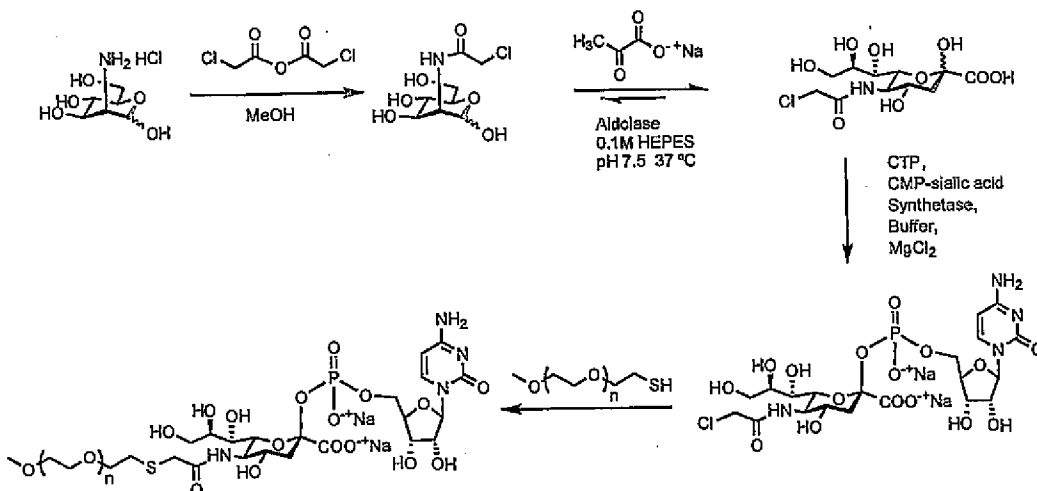
The resulting chloro-derivatized glycan is contacted with pyruvate in the presence of an

aldolase, forming a chloro-derivatized sialic acid. The corresponding nucleotide sugar is

5   prepared by contacted the sialic acid derivative with an appropriate nucleotide triphosphates

and a synthetase. The chloro group on the sialic acid moiety is then displaced with a

nucleophilic PEG derivative, such as thio-PEG.

In a further exemplary embodiment, as shown is Scheme 6, a mannosamine is

acylated with a bis-HOBT dicarboxylate, producing the corresponding amido-alkyl-

10   carboxylic acid, which is subsequently converted to a sialic acid derivative. The sialic acid

derivative is converted to a nucleotide sugar, and the carboxylic acid is activated and reacted

with a nucleophilic PEG derivative, such as amino-PEG.

Scheme 6
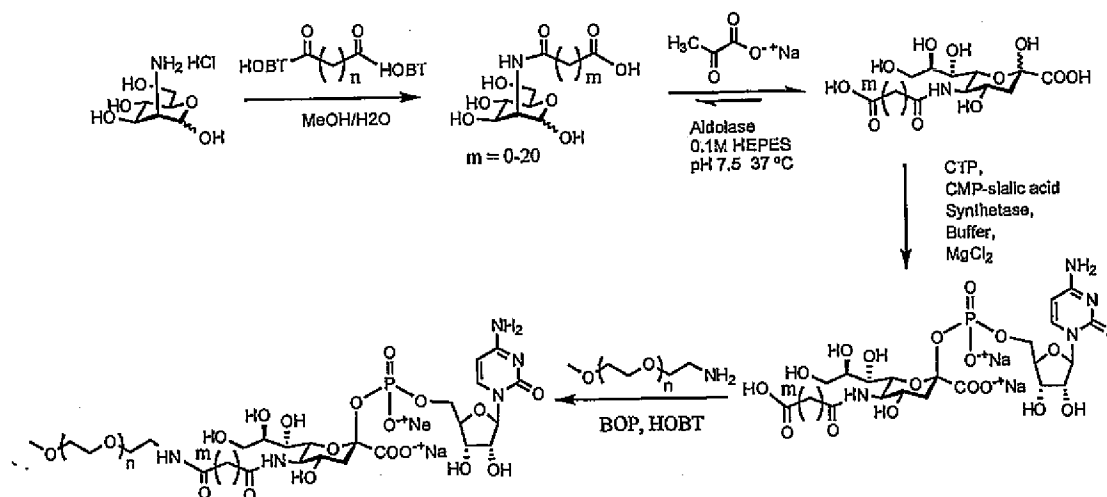
In another exemplary embodiment, set forth in Scheme 7, amine- and carboxyl-
protected neuraminic acid is activated by converting the primary hydroxyl group to the
corresponding p-toluenesulfonate ester, and the methyl ester is cleaved. The activated
neuraminic acid is converted to the corresponding nucleotide sugar, and the activating group
5   is displaced by a nucleophilic PEG species, such as thio-PEG.

Scheme 7
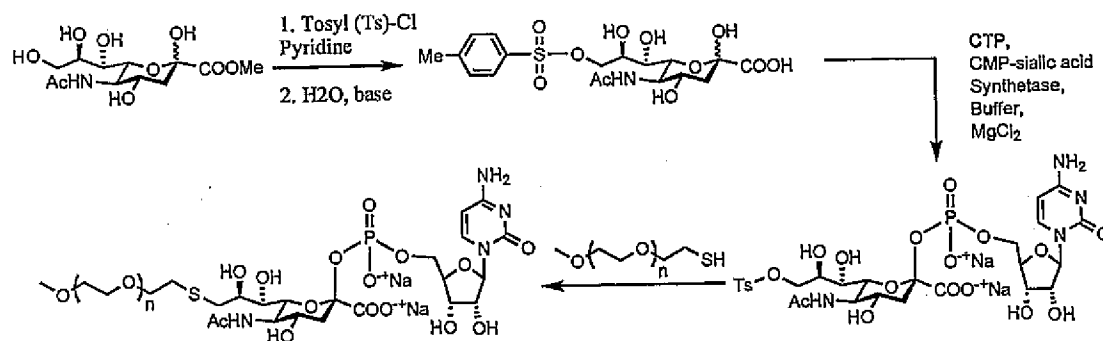
10   In yet a further exemplary embodiment, as set forth in Scheme 8, the primary
hydroxyl moiety of an amine- and carboxyl-protected neuraminic acid derivative is alkylated
using an electrophilic PEG, such as chloro-PEG. The methyl ester is subsequently cleaved
and the PEG-sugar is converted to a nucleotide sugar.

15   **Scheme 8**
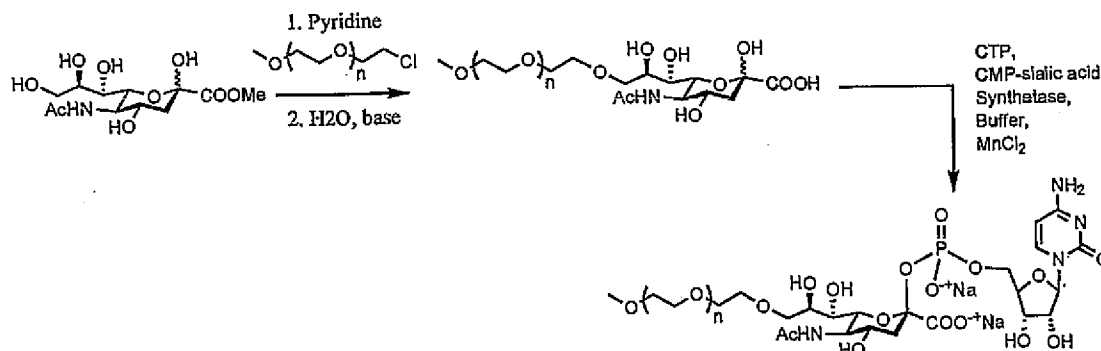
Glycans other than sialic acid can be derivatized with PEG using the methods set forth
herein. The derivatized glycans, themselves, are also within the scope of the invention.

20   Thus, Scheme 9 provides an exemplary synthetic route to a PEGylated galactose nucleotide

sugar. The primary hydroxyl group of galactose is activated as the corresponding toluenesulfonate ester, which is subsequently converted to a nucleotide sugar.

Scheme 9
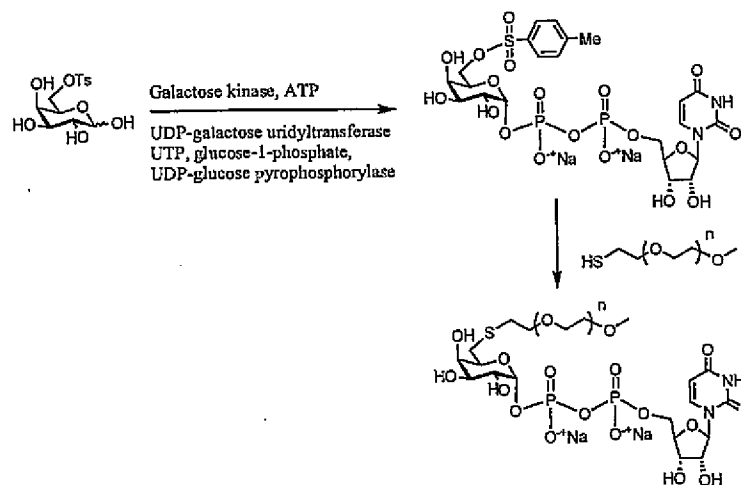
Scheme 10 sets forth an exemplary route for preparing a galactose-PEG derivative that is based upon a galactose-6-amine moiety. Thus, galactosamine is converted to a nucleotide sugar, and the amine moiety of galactosamine is functionalized with an active PEG derivative.
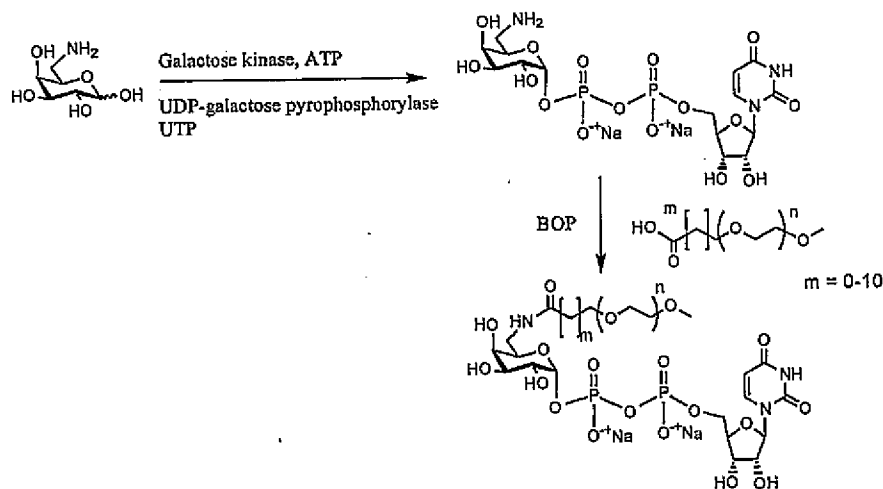
Scheme 10

Scheme 11 provides another exemplary route to galactose derivatives. The starting

point for Scheme 11 is galactose-2-amine, which is converted to a nucleotide sugar. The

amine moiety of the nucleotide sugar is the locus for attaching a PEG derivative, such as

Methoxy-PEG (mPEG) carboxylic acid.

5

Scheme 11
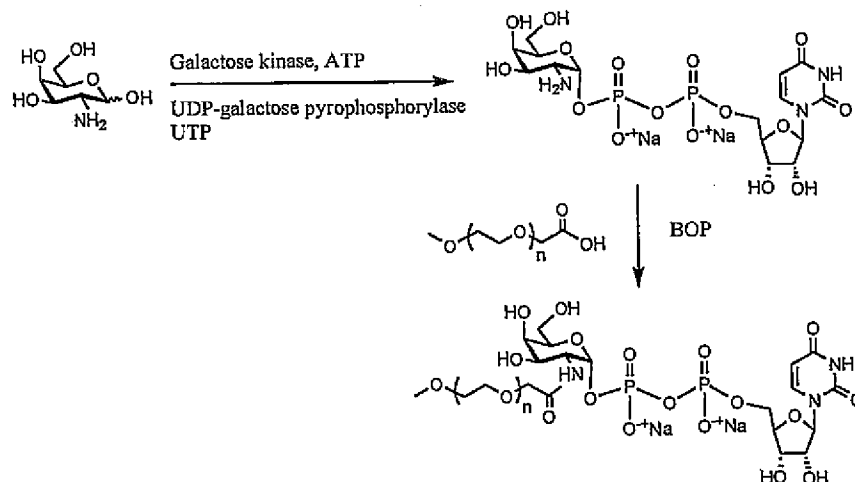
Exemplary moieties attached to the conjugates disclosed herein include, but are not

10   limited to, PEG derivatives (*e.g.*, acyl-PEG, acyl-alkyl-PEG, alkyl-acyl-PEG carbamoyl-

PEG, aryl-PEG, alkyl-PEG), PPG derivatives (*e.g.*, acyl-PPG, acyl-alkyl-PPG, alkyl-acyl-

PPG carbamoyl-PPG, aryl-PPG), polyapartic acid, polyglutamate, polylysine, therapeutic

moieties, diagnostic moieties, mannose-6-phosphate, heparin, heparan, SLe^x, mannose,

mannose-6-phosphate, Sialyl Lewis X, FGF, VFGF, proteins (e.g., transferrin), chondroitin,

15   keratan, dermatan, dextran, modified dextran, amylose, bisphosphate, poly-SA, hyaluronic

acid, keritan, albumin, integrins, antennary oligosaccharides, peptides and the like. Methods

of conjugating the various modifying groups to a saccharide moiety are readily accessible to

those of skill in the art (POLY (ETHYLENE GLYCOL CHEMISTRY : BIOTECHNICAL AND

BIOMEDICAL APPLICATIONS, J. Milton Harris, Ed., Plenum Pub. Corp., 1992; POLY

20   (ETHYLENE GLYCOL) CHEMICAL AND BIOLOGICAL APPLICATIONS, J. Milton Harris, Ed., ACS

Symposium Series No. 680, American Chemical Society, 1997; Hermanson, BIOCONJUGATE

TECHNIQUES, Academic Press, San Diego, 1996; and Dunn *et al.*, Eds. POLYMERIC DRUGS

AND DRUG DELIVERY SYSTEMS, ACS Symposium Series Vol. 469, American Chemical Society, Washington, D.C. 1991).

Purification of sugars, nucleotide sugars and derivatives

The nucleotide sugars and derivatives produced by the above processes can be used

5   without purification. However, it is usually preferred to recover the product. Standard, well-known techniques for recovery of glycosylated saccharides such as thin or thick layer chromatography, column chromatography, ion exchange chromatography, or membrane filtration can be used. It is preferred to use membrane filtration, more preferably utilizing a reverse osmotic membrane, or one or more column chromatographic techniques for the

10   recovery as is discussed hereinafter and in the literature cited herein. For instance, membrane filtration wherein the membranes have molecular weight cutoff of about 3000 to about 10,000 can be used to remove proteins for reagents having a molecular weight of less than 10,000 Da.. Membrane filtration or reverse osmosis can then be used to remove salts and/or purify the product saccharides (*see, e.g.,* WO 98/15581). Nanofilter membranes are a class of

15   reverse osmosis membranes that pass monovalent salts but retain polyvalent salts and uncharged solutes larger than about 100 to about 2,000 Daltons, depending upon the membrane used. Thus, in a typical application, saccharides prepared by the methods of the present invention will be retained in the membrane and contaminating salts will pass through.

20   G. Cross-linking Groups

Preparation of the modified sugar for use in the methods of the present invention includes attachment of a modifying group to a sugar residue and forming a stable adduct, which is a substrate for a glycosyltransferase. Thus, it is often preferred to use a cross-linking agent to conjugate the modifying group and the sugar. Exemplary bifunctional

25   compounds which can be used for attaching modifying groups to carbohydrate moieties include, but are not limited to, bifunctional poly(ethylene glycols), polyamides, polyethers, polyesters and the like. General approaches for linking carbohydrates to other molecules are known in the literature. *See,* for example, Lee *et al., Biochemistry* **28**: 1856 (1989); Bhatia *et al., Anal. Biochem.* **178**: 408 (1989); Janda *et al., J. Am. Chem. Soc.* **112**: 8886 (1990) and

30   Bednarski *et al.,* WO 92/18135. In the discussion that follows, the reactive groups are treated as benign on the sugar moiety of the nascent modified sugar. The focus of the discussion is

for clarity of illustration. Those of skill in the art will appreciate that the discussion is relevant to reactive groups on the modifying group as well.

An exemplary strategy involves incorporation of a protected sulfhydryl onto the sugar using the heterobifunctional crosslinker SPDP (n-succinimidyl-3-(2-pyridyldithio)propionate

5   and then deprotecting the sulfhydryl for formation of a disulfide bond with another sulfhydryl on the modifying group.

If SPDP detrimentally affects the ability of the modified sugar to act as a glycosyltransferase substrate, one of an array of other crosslinkers such as 2-iminothiolane or N-succinimidyl S-acetylthioacetate (SATA) is used to form a disulfide bond. 2-

10   iminothiolane reacts with primary amines, instantly incorporating an unprotected sulfhydryl onto the amine-containing molecule. SATA also reacts with primary amines, but incorporates a protected sulfhydryl, which is later deacetylated using hydroxylamine to produce a free sulfhydryl. In each case, the incorporated sulfhydryl is free to react with other sulfhydryls or protected sulfhydryl, like SPDP, forming the required disulfide bond.

15   The above-described strategy is exemplary, and not limiting, of linkers of use in the invention. Other crosslinkers are available that can be used in different strategies for crosslinking the modifying group to the peptide. For example, TPCH(S-(2-thiopyridyl)-L-cysteine hydrazide and TPMPH ((S-(2-thiopyridyl) mercapto-propionohydrazide) react with carbohydrate moieties that have been previously oxidized by mild periodate treatment, thus

20   forming a hydrazone bond between the hydrazide portion of the crosslinker and the periodate generated aldehydes. TPCH and TPMPH introduce a 2-pyridylthione protected sulfhydryl group onto the sugar, which can be deprotected with DTT and then subsequently used for conjugation, such as forming disulfide bonds between components.

If disulfide bonding is found unsuitable for producing stable modified sugars, other

25   crosslinkers may be used that incorporate more stable bonds between components. The heterobifunctional crosslinkers GMBS (N-gama-malimidobutyryloxy)succinimide) and SMCC (succinimidyl 4-(N-maleimido-methyl)cyclohexane) react with primary amines, thus introducing a maleimide group onto the component. The maleimide group can subsequently react with sulfhydryls on the other component, which can be introduced by previously

30   mentioned crosslinkers, thus forming a stable thioether bond between the components. If steric hindrance between components interferes with either component's activity or the ability

of the modified sugar to act as a glycosyltransferase substrate, crosslinkers can be used which introduce long spacer arms between components and include derivatives of some of the previously mentioned crosslinkers (*i.e.*, SPDP). Thus, there is an abundance of suitable crosslinkers, which are useful; each of which is selected depending on the effects it has on

5   optimal peptide conjugate and modified sugar production.

A variety of reagents are used to modify the components of the modified sugar with intramolecular chemical crosslinks (for reviews of crosslinking reagents and crosslinking procedures see: Wold, F., *Meth. Enzymol.* 25: 623-651, 1972; Weetall, H. H., and Cooney, D. A., In: ENZYMES AS DRUGS. (Holcenberg, and Roberts, eds.) pp. 395-442, Wiley, New York,

10   1981; Ji, T. H., *Meth. Enzymol.* 91: 580-609, 1983; Mattson *et al.*, *Mol. Biol. Rep.* 17: 167-183, 1993, all of which are incorporated herein by reference). Preferred crosslinking reagents are derived from various zero-length, homo-bifunctional, and hetero-bifunctional crosslinking reagents. Zero-length crosslinking reagents include direct conjugation of two intrinsic chemical groups with no introduction of extrinsic material. Agents that catalyze formation of

15   a disulfide bond belong to this category. Another example is reagents that induce condensation of a carboxyl and a primary amino group to form an amide bond such as carbodiimides, ethylchloroformate, Woodward's reagent K (2-ethyl-5-phenylisoxazolium-3'-sulfonate), and carbonyldiimidazole. In addition to these chemical reagents, the enzyme transglutaminase (glutamyl-peptide γ-glutamyltransferase; EC 2.3.2.13) may be used as zero-

20   length crosslinking reagent. This enzyme catalyzes acyl transfer reactions at carboxamide groups of protein-linked glutaminyl residues, usually with a primary amino group as substrate. Preferred homo- and hetero-bifunctional reagents contain two identical or two dissimilar sites, respectively, which may be reactive for amino, sulfhydryl, guanidino, indole, or nonspecific groups.

25

2. Preferred Specific Sites in Crosslinking Reagents
a. Amino-Reactive Groups

In one preferred embodiment, the sites on the cross-linker are amino-reactive groups. Useful non-limiting examples of amino-reactive groups include N-hydroxysuccinimide

30   (NHS) esters, imidoesters, isocyanates, acylhalides, arylazides, p-nitrophenyl esters, aldehydes, and sulfonyl chlorides.

NHS esters react preferentially with the primary (including aromatic) amino groups of a modified sugar component. The imidazole groups of histidines are known to compete with primary amines for reaction, but the reaction products are unstable and readily hydrolyzed. The reaction involves the nucleophilic attack of an amine on the acid carboxyl of an NHS

5   ester to form an amide, releasing the N-hydroxysuccinimide. Thus, the positive charge of the original amino group is lost.

Imidoesters are the most specific acylating reagents for reaction with the amine groups of the modified sugar components. At a pH between 7 and 10, imidoesters react only with primary amines. Primary amines attack imidates nucleophilically to produce an

10   intermediate that breaks down to amidine at high pH or to a new imidate at low pH. The new imidate can react with another primary amine, thus crosslinking two amino groups, a case of a putatively monofunctional imidate reacting bifunctionally. The principal product of reaction with primary amines is an amidine that is a stronger base than the original amine. The positive charge of the original amino group is therefore retained.

15   Isocyanates (and isothiocyanates) react with the primary amines of the modified sugar components to form stable bonds. Their reactions with sulfhydryl, imidazole, and tyrosyl groups give relatively unstable products.

Acylazides are also used as amino-specific reagents in which nucleophilic amines of the affinity component attack acidic carboxyl groups under slightly alkaline conditions, *e.g.*

20   pH 8.5.

Arylhalides such as 1,5-difluoro-2,4-dinitrobenzene react preferentially with the amino groups and tyrosine phenolic groups of modified sugar components, but also with sulfhydryl and imidazole groups.

p-Nitrophenyl esters of mono- and dicarboxylic acids are also useful amino-reactive

25   groups. Although the reagent specificity is not very high, α- and ϵ-amino groups appear to react most rapidly.

Aldehydes such as glutaraldehyde react with primary amines of modified sugar. Although unstable Schiff bases are formed upon reaction of the amino groups with the aldehydes of the aldehydes, glutaraldehyde is capable of modifying the modified sugar with

30   stable crosslinks. At pH 6-8, the pH of typical crosslinking conditions, the cyclic polymers undergo a dehydration to form α-β unsaturated aldehyde polymers. Schiff bases, however,

are stable, when conjugated to another double bond. The resonant interaction of both double bonds prevents hydrolysis of the Schiff linkage. Furthermore, amines at high local concentrations can attack the ethylenic double bond to form a stable Michael addition product.

5   Aromatic sulfonyl chlorides react with a variety of sites of the modified sugar components, but reaction with the amino groups is the most important, resulting in a stable sulfonamide linkage.

b. Sulfhydryl-Reactive Groups

10   In another preferred embodiment, the sites are sulfhydryl-reactive groups. Useful, non-limiting examples of sulfhydryl-reactive groups include maleimides, alkyl halides, pyridyl disulfides, and thiophthalimides.

Maleimides react preferentially with the sulfhydryl group of the modified sugar components to form stable thioether bonds. They also react at a much slower rate with

15   primary amino groups and the imidazole groups of histidines. However, at pH 7 the maleimide group can be considered a sulfhydryl-specific group, since at this pH the reaction rate of simple thiols is 1000-fold greater than that of the corresponding amine.

Alkyl halides react with sulfhydryl groups, sulfides, imidazoles, and amino groups. At neutral to slightly alkaline pH, however, alkyl halides react primarily with sulfhydryl

20   groups to form stable thioether bonds. At higher pH, reaction with amino groups is favored.

Pyridyl disulfides react with free sulfhydryls via disulfide exchange to give mixed disulfides. As a result, pyridyl disulfides are the most specific sulfhydryl-reactive groups.

Thiophthalimides react with free sulfhydryl groups to form disulfides.

25
c. Carboxyl-Reactive Residue

In another embodiment, carbodiimides soluble in both water and organic solvent, are used as carboxyl-reactive reagents. These compounds react with free carboxyl groups forming a pseudourea that can then coupled to available amines yielding an amide linkage. Procedures to modify a carboxyl group with carbodiimide is well know in the art (see,

30   Yamada *et al., Biochemistry* **20**: 4836-4842, 1981).

3. Preferred Nonspecific Sites in Crosslinking Reagents

In addition to the use of site-specific reactive moieties, the present invention contemplates the use of non-specific reactive groups to link the sugar to the modifying group.

Exemplary non-specific cross-linkers include photoactivatable groups, completely
5   inert in the dark, which are converted to reactive species upon absorption of a photon of appropriate energy. In one preferred embodiment, photoactivatable groups are selected from precursors of nitrenes generated upon heating or photolysis of azides. Electron-deficient nitrenes are extremely reactive and can react with a variety of chemical bonds including N-H, O-H, C-H, and C=C. Although three types of azides (aryl, alkyl, and acyl derivatives) may
10 be employed, arylazides are presently preferred. The reactivity of arylazides upon photolysis is better with N-H and O-H than C-H bonds. Electron-deficient arylnitrenes rapidly ring-expand to form dehydroazepines, which tend to react with nucleophiles, rather than form C-H insertion products. The reactivity of arylazides can be increased by the presence of electron-withdrawing substituents such as nitro or hydroxyl groups in the ring. Such substituents push
15 the absorption maximum of arylazides to longer wavelength. Unsubstituted arylazides have an absorption maximum in the range of 260-280 nm, while hydroxy and nitroarylazides absorb significant light beyond 305 nm. Therefore, hydroxy and nitroarylazides are most preferable since they allow to employ less harmful photolysis conditions for the affinity component than unsubstituted arylazides.

20   In another preferred embodiment, photoactivatable groups are selected from fluorinated arylazides. The photolysis products of fluorinated arylazides are arylnitrenes, all of which undergo the characteristic reactions of this group, including C-H bond insertion, with high efficiency (Keana et al., J. Org. Chem. 55: 3640-3647, 1990).

In another embodiment, photoactivatable groups are selected from benzophenone
25 residues. Benzophenone reagents generally give higher crosslinking yields than arylazide reagents.

In another embodiment, photoactivatable groups are selected from diazo compounds, which form an electron-deficient carbene upon photolysis. These carbenes undergo a variety of reactions including insertion into C-H bonds, addition to double bonds (including aromatic
30 systems), hydrogen attraction and coordination to nucleophilic centers to give carbon ions.

In still another embodiment, photoactivatable groups are selected from diazopyruvates. For example, the p-nitrophenyl ester of p-nitrophenyl diazopyruvate reacts with aliphatic amines to give diazopyruvic acid amides that undergo ultraviolet photolysis to form aldehydes. The photolyzed diazopyruvate-modified affinity component will react like

5   formaldehyde or glutaraldehyde forming crosslinks.

4. Homobifunctional Reagents

a. Homobifunctional crosslinkers reactive with primary amines

Synthesis, properties, and applications of amine-reactive cross-linkers are

10   commercially described in the literature (for reviews of crosslinking procedures and reagents, *see above*). Many reagents are available (*e.g.*, Pierce Chemical Company, Rockford, Ill.; Sigma Chemical Company, St. Louis, Mo.; Molecular Probes, Inc., Eugene, OR.).

Preferred, non-limiting examples of homobifunctional NHS esters include disuccinimidyl glutarate (DSG), disuccinimidyl suberate (DSS), bis(sulfosuccinimidyl)

15   suberate (BS), disuccinimidyl tartarate (DST), disulfosuccinimidyl tartarate (sulfo-DST), bis-2-(succinimidooxycarbonyloxy)ethylsulfone (BSOCOES), bis-2-(sulfosuccinimidooxy-carbonyloxy)ethylsulfone (sulfo-BSOCOES), ethylene glycolbis(succinimidylsuccinate) (EGS), ethylene glycolbis(sulfosuccinimidylsuccinate) (sulfo-EGS), dithiobis(succinimidyl-propionate (DSP), and dithiobis(sulfosuccinimidylpropionate (sulfo-DSP). Preferred, non-

20   limiting examples of homobifunctional imidoesters include dimethyl malonimidate (DMM), dimethyl succinimidate (DMSC), dimethyl adipimidate (DMA), dimethyl pimelimidate (DMP), dimethyl suberimidate (DMS), dimethyl-3,3'-oxydipropionimidate (DODP), dimethyl-3,3'-(methylenedioxy)dipropionimidate (DMDP), dimethyl-,3'-(dimethylenedioxy)dipropionimidate (DDDP), dimethyl-3,3'-(tetramethylenedioxy)-

25   dipropionimidate (DTDP), and dimethyl-3,3'-dithiobispropionimidate (DTBP).

Preferred, non-limiting examples of homobifunctional isothiocyanates include: p-phenylenediisothiocyanate (DITC), and 4,4'-diisothiocyano-2,2'-disulfonic acid stilbene (DIDS).

Preferred, non-limiting examples of homobifunctional isocyanates include xylene-

30   diisocyanate, toluene-2,4-diisocyanate, toluene-2-isocyanate-4-isothiocyanate, 3-

methoxydiphenylmethane-4,4'-diisocyanate, 2,2'-dicarboxy-4,4'-azophenyldiisocyanate, and hexamethylenediisocyanate.

Preferred, non-limiting examples of homobifunctional arylhalides include 1,5-difluoro-2,4-dinitrobenzene (DFDNB), and 4,4'-difluoro-3,3'-dinitrophenyl-sulfone.

5   Preferred, non-limiting examples of homobifunctional aliphatic aldehyde reagents include glyoxal, malondialdehyde, and glutaraldehyde.

Preferred, non-limiting examples of homobifunctional acylating reagents include nitrophenyl esters of dicarboxylic acids.

Preferred, non-limiting examples of homobifunctional aromatic sulfonyl chlorides

10   include phenol-2,4-disulfonyl chloride, and α-naphthol-2,4-disulfonyl chloride.

Preferred, non-limiting examples of additional amino-reactive homobifunctional reagents include erythritolbiscarbonate which reacts with amines to give biscarbamates.

b. Homobifunctional Crosslinkers Reactive with Free Sulfhydryl

15   Groups

Synthesis, properties, and applications of such reagents are described in the literature (for reviews of crosslinking procedures and reagents, *see above*). Many of the reagents are commercially available (*e.g.*, Pierce Chemical Company, Rockford, Ill.; Sigma Chemical Company, St. Louis, Mo.; Molecular Probes, Inc., Eugene, OR).

20   Preferred, non-limiting examples of homobifunctional maleimides include bismaleimidohexane (BMH), N,N'-(1,3-phenylene) bismaleimide, N,N'-(1,2-phenylene)bismaleimide, azophenyldimaleimide, and bis(N-maleimidomethyl)ether.

Preferred, non-limiting examples of homobifunctional pyridyl disulfides include 1,4-di-3'-(2'-pyridyldithio)propionamidobutane (DPDPB).

25   Preferred, non-limiting examples of homobifunctional alkyl halides include 2,2'-dicarboxy-4,4'-diiodoacetamidoazobenzene, α,α'-diiodo-p-xylenesulfonic acid, α, α'-dibromo-p-xylenesulfonic acid, N,N'-bis(b-bromoethyl)benzylamine, N,N'-di(bromoacetyl)phenylthydrazine, and 1,2-di(bromoacetyl)amino-3-phenylpropane.

c. Homobifunctional Photoactivatable Crosslinkers

Synthesis, properties, and applications of such reagents are described in the literature (for reviews of crosslinking procedures and reagents, *see above*). Some of the reagents are commercially available (*e.g.*, Pierce Chemical Company, Rockford, Ill.; Sigma Chemical Company, St. Louis, Mo.; Molecular Probes, Inc., Eugene, OR).

Preferred, non-limiting examples of homobifunctional photoactivatable crosslinker include bis-β-(4-azidosalicylamido)ethyldisulfide (BASED), di-N-(2-nitro-4-azidophenyl)-cystamine-S,S-dioxide (DNCO), and 4,4'-dithiobisphenylazide.

5. HeteroBifunctional Reagents

a. Amino-Reactive HeteroBifunctional Reagents with a Pyridyl Disulfide Moiety

Synthesis, properties, and applications of such reagents are described in the literature (for reviews of crosslinking procedures and reagents, *see above*). Many of the reagents are commercially available (*e.g.*, Pierce Chemical Company, Rockford, Ill.; Sigma Chemical Company, St. Louis, Mo.; Molecular Probes, Inc., Eugene, OR).

Preferred, non-limiting examples of hetero-bifunctional reagents with a pyridyl disulfide moiety and an amino-reactive NHS ester include N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), succinimidyl 6-3-(2-pyridyldithio)propionamidohexanoate (LC-SPDP), sulfosuccinimidyl 6-3-(2-pyridyldithio)propionamidohexanoate (sulfo-LCSPDP), 4-succinimidyloxycarbonyl-α-methyl-α-(2-pyridyldithio)toluene (SMPT), and sulfosuccinimidyl 6-α-methyl-α-(2-pyridyldithio)toluamidohexanoate (sulfo-LC-SMPT).

b. Amino-Reactive HeteroBifunctional Reagents with a Maleimide Moiety

Synthesis, properties, and applications of such reagents are described in the literature. Preferred, non-limiting examples of hetero-bifunctional reagents with a maleimide moiety and an amino-reactive NHS ester include succinimidyl maleimidylacetate (AMAS), succinimidyl 3-maleimidylpropionate (BMPS), N- γ-maleimidobutyryloxysuccinimide ester (GMBS)N-γ-maleimidobutyryloxysulfo succinimide ester (sulfo-GMBS) succinimidyl 6-maleimidylhexanoate (EMCS), succinimidyl 3-maleimidylbenzoate (SMB),

m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester (sulfo-MBS), succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC), sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC), succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB), and

5   sulfosuccinimidyl 4-(p-maleimidophenyl)butyrate (sulfo-SMPB).

c. Amino-Reactive HeteroBifunctional Reagents with an Alkyl Halide Moiety

Synthesis, properties, and applications of such reagents are described in the literature.

10   Preferred, non-limiting examples of hetero-bifunctional reagents with an alkyl halide moiety and an amino-reactive NHS ester include N-succinimidyl-(4-iodoacetyl)aminobenzoate (SIAB), sulfosuccinimidyl-(4-iodoacetyl)aminobenzoate (sulfo-SIAB), succinimidyl-6-(iodoacetyl)aminohexanoate (SIAX), succinimidyl-6-(6-((iodoacetyl)-amino)hexanoylamino)hexanoate (SIAXX), succinimidyl-6-(((4-(iodoacetyl)-amino)-

15   methyl)-cyclohexane-1-carbonyl)aminohexanoate (SIACX), and succinimidyl-4((iodoacetyl)-amino)methylcyclohexane-1-carboxylate (SIAC).

A preferred example of a hetero-bifunctional reagent with an amino-reactive NHS ester and an alkyl dihalide moiety is N-hydroxysuccinimidyl 2,3-dibromopropionate (SDBP). SDBP introduces intramolecular crosslinks to the affinity component by conjugating its

20   amino groups. The reactivity of the dibromopropionyl moiety towards primary amine groups is controlled by the reaction temperature (McKenzie *et al.*, *Protein Chem.* 7: 581-592 (1988)).

Preferred, non-limiting examples of hetero-bifunctional reagents with an alkyl halide moiety and an amino-reactive p-nitrophenyl ester moiety include p-nitrophenyl iodoacetate

25   (NPIA).

Other cross-linking agents are known to those of skill in the art. *See*, for example, Pomato *et al.*, U.S. Patent No. 5,965,106. It is within the abilities of one of skill in the art to choose an appropriate cross-linking agent for a particular application.

d. Cleavable Linker Groups

In yet a further embodiment, the linker group is provided with a group that can be cleaved to release the modifying group from the sugar residue. Many cleavable groups are known in the art. *See,* for example, Jung *et al., Biochem. Biophys. Acta* 761: 152-162 (1983);

5 Joshi *et al., J. Biol. Chem.* 265: 14518-14525 (1990); Zarling *et al., J. Immunol.* 124: 913-920 (1980); Bouizar *et al., Eur. J. Biochem.* 155: 141-147 (1986); Park *et al., J. Biol. Chem.* 261: 205-210 (1986); Browning *et al., J. Immunol.* 143: 1859-1867 (1989). Moreover a broad range of cleavable, bifunctional (both homo- and hetero-bifunctional) linker groups is commercially available from suppliers such as Pierce.

10 Exemplary cleavable moieties can be cleaved using light, heat or reagents such as thiols, hydroxylamine, bases, periodate and the like. Moreover, certain preferred groups are cleaved *in vivo* in response to being endocytosed (*e.g.,* cis-aconityl; *see,* Shen *et al., Biochem. Biophys. Res. Commun.* 102: 1048 (1991)). Preferred cleavable groups comprise a cleavable moiety which is a member selected from the group consisting of disulfide, ester, imide,

15 carbonate, nitrobenzyl, phenacyl and benzoin groups.

e. Conjugation of Modified Sugars to Peptides

The modified sugars are conjugated to a glycosylated or non-glycosylated peptide using an appropriate enzyme to mediate the conjugation. Preferably, the concentrations of

20 the modified donor sugar(s), enzyme(s) and acceptor peptide(s) are selected such that glycosylation proceeds until the acceptor is consumed. The considerations discussed below, while set forth in the context of a sialyltransferase, are generally applicable to other glycosyltransferase reactions.

A number of methods of using glycosyltransferases to synthesize desired

25 oligosaccharide structures are known and are generally applicable to the instant invention. Exemplary methods are described, for instance, WO 96/32491, Ito *et al., Pure Appl. Chem.* 65: 753 (1993), and U.S. Pat. Nos. 5,352,670, 5,374,541, and 5,545,553.

The present invention is practiced using a single glycosyltransferase or a combination of glycosyltransferases. For example, one can use a combination of a sialyltransferase and a

30 galactosyltransferase. In those embodiments using more than one enzyme, the enzymes and substrates are preferably combined in an initial reaction mixture, or the enzymes and reagents

for a second enzymatic reaction are added to the reaction medium once the first enzymatic

reaction is complete or nearly complete. By conducting two enzymatic reactions in sequence

in a single vessel, overall yields are improved over procedures in which an intermediate

species is isolated. Moreover, cleanup and disposal of extra solvents and by-products is

5   reduced.

In a preferred embodiment, each of the first and second enzyme is a

glycosyltransferase. In another preferred embodiment, one enzyme is an endoglycosidase. In

another preferred embodiment, one enzyme is an exoglycosidase. In an additional preferred

embodiment, more than two enzymes are used to assemble the modified glycoprotein of the

10   invention. The enzymes are used to alter a saccharide structure on the peptide at any point

either before or after the addition of the modified sugar to the peptide.

In another embodiment, at least two of the enzymes are glycosyltransferases and the

last sugar added to the saccharide structure of the peptide is a non-modified sugar. Instead,

the modified sugar is internal to the glycan structure and therefore need not be the ultimate

15   sugar on the glycan. In an exemplary embodiment, galactosyltransferase may catalyze the

transfer of Gal-PEG from UDP-Gal-PEG onto the glycan, followed by incubation in the

presence of ST3Gal3 and CMP-SA, which serves to add a "capping" unmodified sialic acid

onto the glycan (Figure 23A).

In another embodiment, at least two of the enzymes used are glycosyltransferases, and

20   at least two modified sugars are added to the glycan structures on the peptide. In this manner,

two or more different glycoconjugates may be added to one or more glycans on a peptide.

This process generates glycan structures having two or more functionally different modified

sugars. In an exemplary embodiment, incubation of the peptide with GnT-I, II and UDP-

GlcNAc-PEG serves to add a GlcNAc-PEG molecule to the glycan; incubation with

25   galactosyltransferase and UDP-Gal then serves to add a Gal residue thereto; and, incubation

with ST3Gal3 and CMP-SA-Man-6-Phosphate serves to add a SA-mannose-6-phosphate

molecule to the glycan. This series of reactions results in a glycan chain having the

functional characteristics of a PEGylated glycan as well as mannose-6-phosphate targeting

activity (Figure 23B).

30   In another embodiment, at least two of the enzymes used in the reaction are

glycosyltransferases, and again, different modified sugars are added to N-linked and O-

linked glycans on the peptide. This embodiment is useful when two different modified sugars are to be added to the glycans of a peptide, but when it is important to spatially separate the modified sugars on the peptide from each other. For example, if the modified sugars comprise bulky molecules, including but not limited to, PEG and other molecules such as a linker molecule, this method may be preferable. The modified sugars may be added simultaneously to the glycan structures on a peptide, or they may be added sequentially. In an exemplary embodiment, incubation with ST3Gal3 and CMP-SA-PEG serves to add sialic acid-PEG to the N-linked glycans, while incubation with ST3Gal1 and CMP-SA-bisPhosphonate serves to add sialic acid-BisPhosphonate to the O-linked glycans (Figure 23C).

In another embodiment, the method makes use of one or more exo- or endoglycosidase. The glycosidase is typically a mutant, which is engineered to form glycosyl bonds rather than rupture them. The mutant glycanase, sometimes called a glycosynthase, typically includes a substitution of an amino acid residue for an active site acidic amino acid residue. For example, when the endoglycanase is endo-H, the substituted active site residues will typically be Asp at position 130, Glu at position 132 or a combination thereof. The amino acids are generally replaced with serine, alanine, asparagine, or glutamine. Exoglycosidases such as transialylidase are also useful.

The mutant enzyme catalyzes the reaction, usually by a synthesis step that is analogous to the reverse reaction of the endoglycanase hydrolysis step. In these embodiments, the glycosyl donor molecule (*e.g.*, a desired oligo- or mono-saccharide structure) contains a leaving group and the reaction proceeds with the addition of the donor molecule to a GlcNAc residue on the protein. For example, the leaving group can be a halogen, such as fluoride. In other embodiments, the leaving group is a Asn, or a Asn-peptide moiety. In yet further embodiments, the GlcNAc residue on the glycosyl donor molecule is modified. For example, the GlcNAc residue may comprise a 1,2 oxazoline moiety.

In a preferred embodiment, each of the enzymes utilized to produce a conjugate of the invention are present in a catalytic amount. The catalytic amount of a particular enzyme varies according to the concentration of that enzyme's substrate as well as to reaction conditions such as temperature, time and pH value. Means for determining the catalytic

amount for a given enzyme under preselected substrate concentrations and reaction conditions are well known to those of skill in the art.

The temperature at which an above-described process is carried out can range from just above freezing to the temperature at which the most sensitive enzyme denatures.

5 Preferred temperature ranges are about 0 °C to about 55 °C, and more preferably about 20 °C to about 37 °C. In another exemplary embodiment, one or more components of the present method are conducted at an elevated temperature using a thermophilic enzyme.

The reaction mixture is maintained for a period of time sufficient for the acceptor to be glycosylated, thereby forming the desired conjugate. Some of the conjugate can often be

10 detected after a few hours, with recoverable amounts usually being obtained within 24 hours or less. Those of skill in the art understand that the rate of reaction is dependent on a number of variable factors (e.g, enzyme concentration, donor concentration, acceptor concentration, temperature, solvent volume), which are optimized for a selected system.

The present invention also provides for the industrial-scale production of modified

15 peptides. As used herein, an industrial scale generally produces at least one gram of finished, purified conjugate.

In the discussion that follows, the invention is exemplified by the conjugation of modified sialic acid moieties to a glycosylated peptide. The exemplary modified sialic acid is labeled with PEG. The focus of the following discussion on the use of PEG-modified sialic

20 acid and glycosylated peptides is for clarity of illustration and is not intended to imply that the invention is limited to the conjugation of these two partners. One of skill understands that the discussion is generally applicable to the additions of modified glycosyl moieties other than sialic acid. Moreover, the discussion is equally applicable to the modification of a glycosyl unit with agents other than PEG including other water-soluble polymers, therapeutic

25 moieties, and biomolecules.

An enzymatic approach can be used for the selective introduction of PEGylated or PPGylated carbohydrates onto a peptide or glycopeptide. The method utilizes modified sugars containing PEG, PPG, or a masked reactive functional group, and is combined with the appropriate glycosyltransferase or glycosynthase. By selecting the glycosyltransferase

30 that will make the desired carbohydrate linkage and utilizing the modified sugar as the donor substrate, the PEG or PPG can be introduced directly onto the peptide backbone, onto

existing sugar residues of a glycopeptide or onto sugar residues that have been added to a peptide.

An acceptor for the sialyltransferase is present on the peptide to be modified by the methods of the present invention either as a naturally occurring structure or one placed there

5   recombinantly, enzymatically or chemically. Suitable acceptors, include, for example, galactosyl acceptors such as Galβ1,4GlcNAc, Galβ1,4GalNAc, Galβ1,3GalNAc, lacto-N-tetraose, Galβ1,3GlcNAc, Galβ1,3Ara, Galβ1,6GlcNAc, Galβ1,4Glc (lactose), and other acceptors known to those of skill in the art (see, e.g., Paulson et al., J. Biol. Chem. 253: 5617-5624 (1978)).

10   In one embodiment, an acceptor for the sialyltransferase is present on the peptide to be modified upon in vivo synthesis of the peptide. Such peptides can be sialylated using the claimed methods without prior modification of the glycosylation pattern of the peptide. Alternatively, the methods of the invention can be used to sialylate a peptide that does not include a suitable acceptor; one first modifies the peptide to include an acceptor by methods

15   known to those of skill in the art. In an exemplary embodiment, a GalNAc residue is added by the action of a GalNAc transferase.

In an exemplary embodiment, the galactosyl acceptor is assembled by attaching a galactose residue to an appropriate acceptor linked to the peptide, e.g., a GlcNAc. The method includes incubating the peptide to be modified with a reaction mixture that contains a

20   suitable amount of a galactosyltransferase (e.g., galβ1,3 or galβ1,4), and a suitable galactosyl donor (e.g., UDP-galactose). The reaction is allowed to proceed substantially to completion or, alternatively, the reaction is terminated when a preselected amount of the galactose residue is added. Other methods of assembling a selected saccharide acceptor will be apparent to those of skill in the art.

25   In yet another embodiment, peptide-linked oligosaccharides are first "trimmed," either in whole or in part, to expose either an acceptor for the sialyltransferase or a moiety to which one or more appropriate residues can be added to obtain a suitable acceptor. Enzymes such as glycosyltransferases and endoglycosidases (see, for example U.S. Patent No. 5,716,812) are useful for the attaching and trimming reactions. A detailed discussion of

30   "trimming" and remodeling N-linked and O-linked glycans is provided elsewhere herein.

In the discussion that follows, the method of the invention is exemplified by the use of modified sugars having a water-soluble polymer attached thereto. The focus of the discussion is for clarity of illustration. Those of skill will appreciate that the discussion is equally relevant to those embodiments in which the modified sugar bears a therapeutic

5   moiety, biomolecule or the like.

An exemplary embodiment of the invention in which a carbohydrate residue is "trimmed" prior to the addition of the modified sugar is set forth in Figure 14, which sets forth a scheme in which high mannose is trimmed back to the first generation biantennary structure. A modified sugar bearing a water-soluble polymer is conjugated to one or more of

10   the sugar residues exposed by the "trimming back." In one example, a water-soluble polymer is added via a GlcNAc moiety conjugated to the water-soluble polymer. The modified GlcNAc is attached to one or both of the terminal mannose residues of the biantennary structure. Alternatively, an unmodified GlcNAc can be added to one or both of the termini of the branched species.

15   In another exemplary embodiment, a water-soluble polymer is added to one or both of the terminal mannose residues of the biantennary structure via a modified sugar having a galactose residue, which is conjugated to a GlcNAc residue added onto the terminal mannose residues. Alternatively, an unmodified Gal can be added to one or both terminal GlcNAc residues.

20   In yet a further example, a water-soluble polymer is added onto a Gal residue using a modified sialic acid.

Another exemplary embodiment is set forth in Figure 15, which displays a scheme similar to that shown in Figure 14, in which the high mannose structure is "trimmed back" to the mannose from which the biantennary structure branches. In one example, a water-soluble

25   polymer is added via a GlcNAc modified with the polymer. Alternatively, an unmodified GlcNAc is added to the mannose, followed by a Gal with an attached water-soluble polymer. In yet another embodiment, unmodified GlcNAc and Gal residues are sequentially added to the mannose, followed by a sialic acid moiety modified with a water-soluble polymer.

Figure 16 sets forth a further exemplary embodiment using a scheme similar to that

30   shown in Figure 14, in which high mannose is "trimmed back" to the GlcNAc to which the first mannose is attached. The GlcNAc is conjugated to a Gal residue bearing a water-soluble

-183-

polymer. Alternatively, an unmodified Gal is added to the GlcNAc, followed by the addition

of a sialic acid modified with a water-soluble sugar. In yet a further example, the terminal

GlcNAc is conjugated with Gal and the GlcNAc is subsequently fucosylated with a modified

fucose bearing a water-soluble polymer.

5   Figure 17 is a scheme similar to that shown in Figure 14, in which high mannose is

trimmed back to the first GlcNAc attached to the Asn of the peptide. In one example, the

GlcNAc of the GlcNAc-(Fuc)$_a$ residue is conjugated with a GlcNAc bearing a water soluble

polymer. In another example, the GlcNAc of the GlcNAc-(Fuc)$_a$ residue is modified with

Gal, which bears a water soluble polymer. In a still further embodiment, the GlcNAc is

10   modified with Gal, followed by conjugation to the Gal of a sialic acid modified with a water-

soluble polymer.

        Other exemplary embodiments are set forth in Figures 18-22. An illustration of the

array of reaction types with which the present invention may be practiced is provided in each

of the aforementioned figures.

15   The Examples set forth above provide an illustration of the power of the methods set

forth herein. Using the methods of the invention, it is possible to "trim back" and build up a

carbohydrate residue of substantially any desired structure. The modified sugar can be added

to the termini of the carbohydrate moiety as set forth above, or it can be intermediate between

the peptide core and the terminus of the carbohydrate.

20   In an exemplary embodiment, an existing sialic acid is removed from a glycopeptide

using a sialidase, thereby unmasking all or most of the underlying galactosyl residues.

Alternatively, a peptide or glycopeptide is labeled with galactose residues, or an

oligosaccharide residue that terminates in a galactose unit. Following the exposure of or

addition of the galactose residues, an appropriate sialyltransferase is used to add a modified

25   sialic acid. The approach is summarized in Scheme 12.

Scheme 12

CMP-SA-5-NHCOCH₂NH—**PEG(PPG)**.
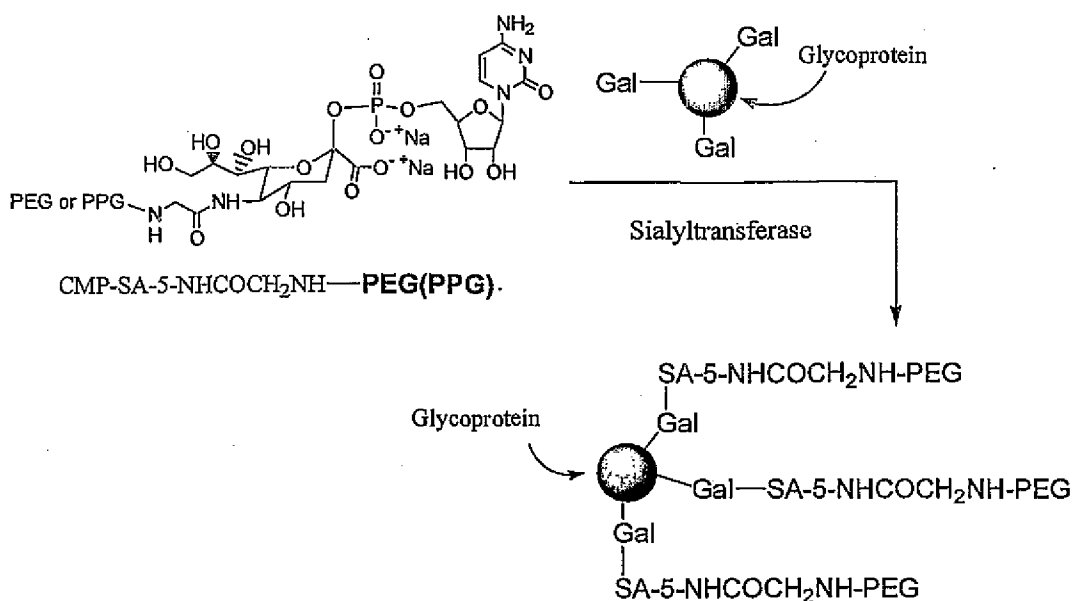
In yet a further approach, summarized in Scheme 13, a masked reactive functionality
5   is present on the sialic acid. The masked reactive group is preferably unaffected by the
conditions used to attach the modified sialic acid to the peptide. After the covalent
attachment of the modified sialic acid to the peptide, the mask is removed and the peptide is
conjugated with an agent such as PEG, PPG, a therapeutic moiety, biomolecule or other
agent. The agent is conjugated to the peptide in a specific manner by its reaction with the
10   unmasked reactive group on the modified sugar residue.

Scheme 13
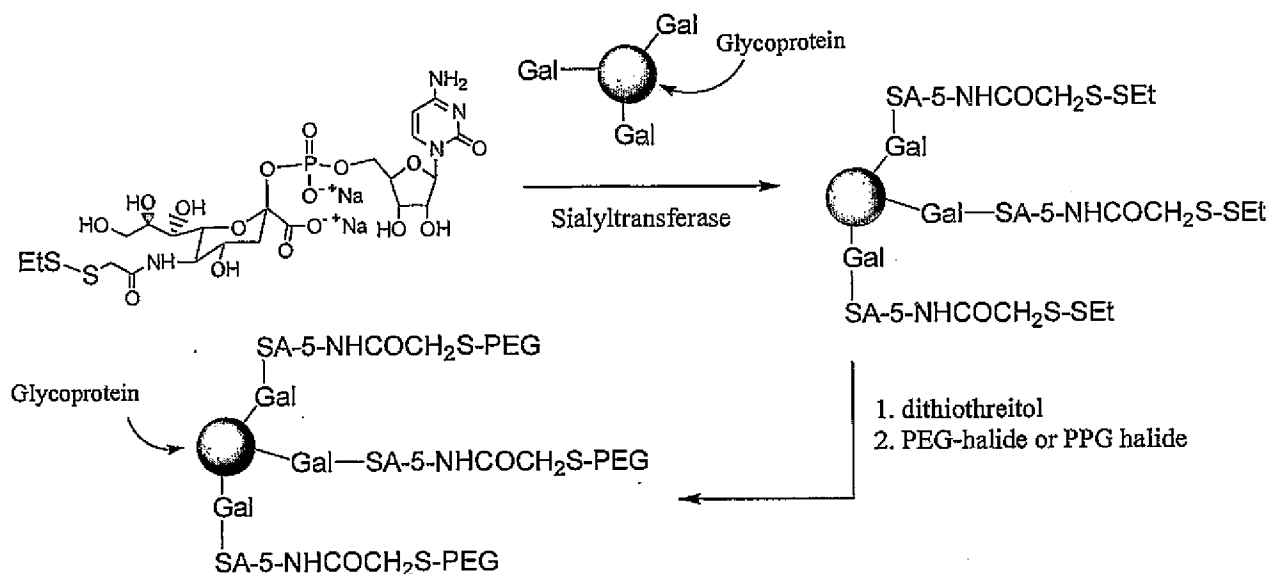
5   Any modified sugar can be used with its appropriate glycosyltransferase, depending
on the terminal sugars of the oligosaccharide side chains of the glycopeptide (Table 4). As
discussed above, the terminal sugar of the glycopeptide required for introduction of the
PEGylated or PPGylated structure can be introduced naturally during expression or it can be
produced post expression using the appropriate glycosidase(s), glycosyltransferase(s) or mix
10   of glycosidase(s) and glycosyltransferase(s).

Table 4. Modified sugars.

UDP-galactose-derivatives

UDP-galactosamine-derivatives
(when A = NH, R_4 may be acetyl)

UDP-Glucose-derivatives

UDP-Glucosamine-derivatives
(when A = NH, R_4 may be acetyl)

GDP-Mannose-derivatives

GDP-fucose-derivatives
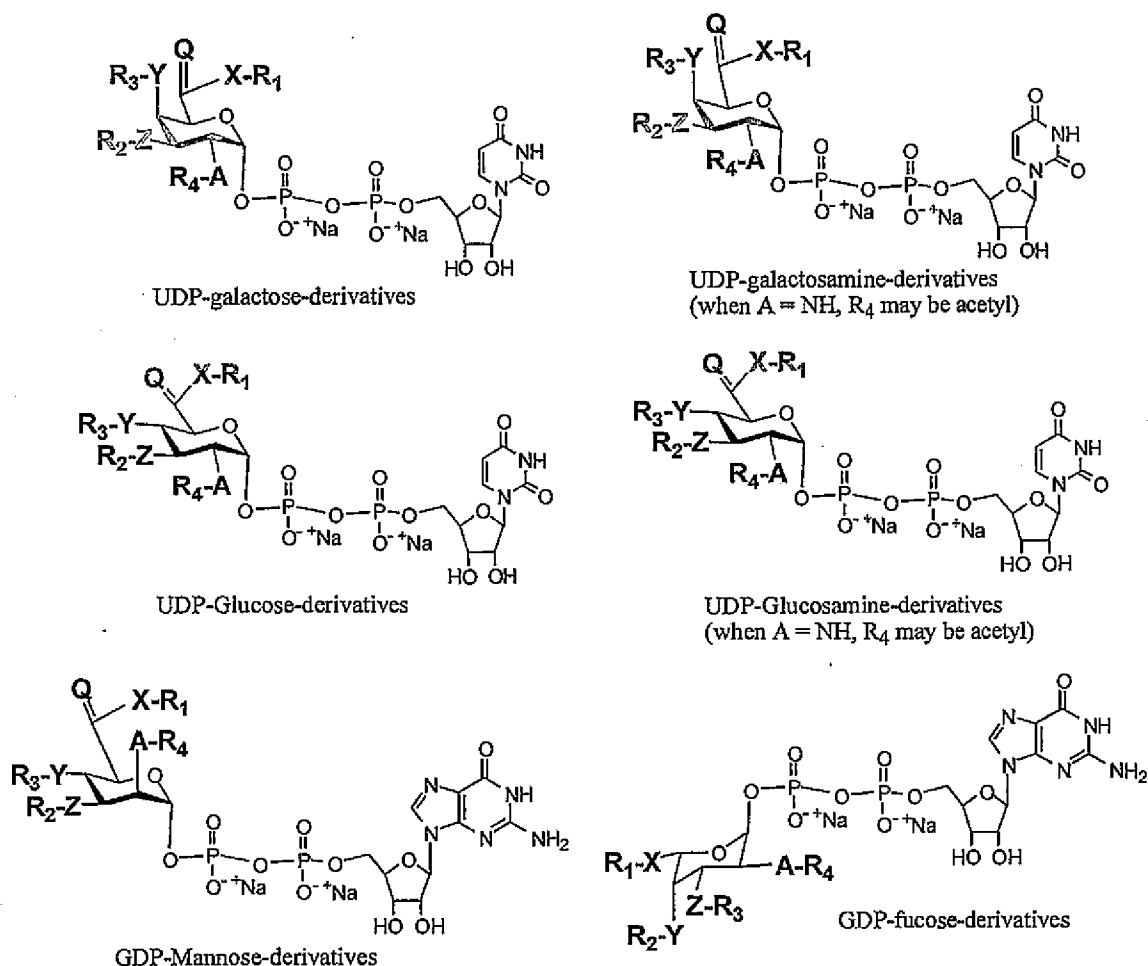
X = O, NH, S, CH_2, N-$(R_{1-5})_2$. Y = X; Z = X; A = X; B = X. Q = H_2, O, S, NH, N-R. R, R_{1-4} = H, Linker-M, M. M = Ligand of interest	Ligand of interest = acyl-PEG, acyl-PPG, alkyl-PEG, acyl-alkyl-PEG, acyl-alkyl-PEG, carbamoyl-PEG, carbamoyl-PPG, PEG, PPG, acyl-aryl-PEG, acyl-aryl-PPG, aryl-PEG, aryl-PPG, Mannose-6-phosphate, heparin, heparan, SLex, Mannose, FGF, VFGF, protein, chondroitin, keratan, dermatan, albumin, integrins, peptides, etc.

In a further exemplary embodiment, UDP-galactose-PEG is reacted with bovine milk
5   β1,4-galactosyltransferase, thereby transferring the modified galactose to the appropriate

terminal N-acetylglucosamine structure. The terminal GlcNAc residues on the glycopeptide

may be produced during expression, as may occur in such expression systems as mammalian,

insect, plant or fungus, but also can be produced by treating the glycopeptide with a sialidase

and/or glycosidase and/or glycosyltransferase, as required.

In another exemplary embodiment, a GlcNAc transferase, such as GnT-I-IV, is utilized to transfer PEGylated-GlcNc to a mannose residue on a glycopeptide. In a still further exemplary embodiment, the N- and/or O-linked glycan structures are enzymatically removed from a glycopeptide to expose an amino acid or a terminal glycosyl residue that is

5   subsequently conjugated with the modified sugar. For example, an endoglycanase is used to remove the N-linked structures of a glycopeptide to expose a terminal GlcNAc as a GlcNAc-linked-Asn on the glycopeptide. UDP-Gal-PEG and the appropriate galactosyltransferase is used to introduce the PEG- or PPG-galactose functionality onto the exposed GlcNAc.

In an alternative embodiment, the modified sugar is added directly to the peptide

10   backbone using a glycosyltransferase known to transfer sugar residues to the peptide backbone. This exemplary embodiment is set forth in Scheme 14. Exemplary glycosyltransferases useful in practicing the present invention include, but are not limited to, GalNAc transferases (GalNAc T1-14), GlcNAc transferases, fucosyltransferases, glucosyltransferases, xylosyltransferases, mannosyltransferases and the like. Use of this

15   approach allows the direct addition of modified sugars onto peptides that lack any carbohydrates or, alternatively, onto existing glycopeptides. In both cases, the addition of the modified sugar occurs at specific positions on the peptide backbone as defined by the substrate specificity of the glycosyltransferase and not in a random manner as occurs during modification of a protein's peptide backbone using chemical methods. An array of agents

20   can be introduced into proteins or glycopeptides that lack the glycosyltransferase substrate peptide sequence by engineering the appropriate amino acid sequence into the peptide chain.

Scheme 14
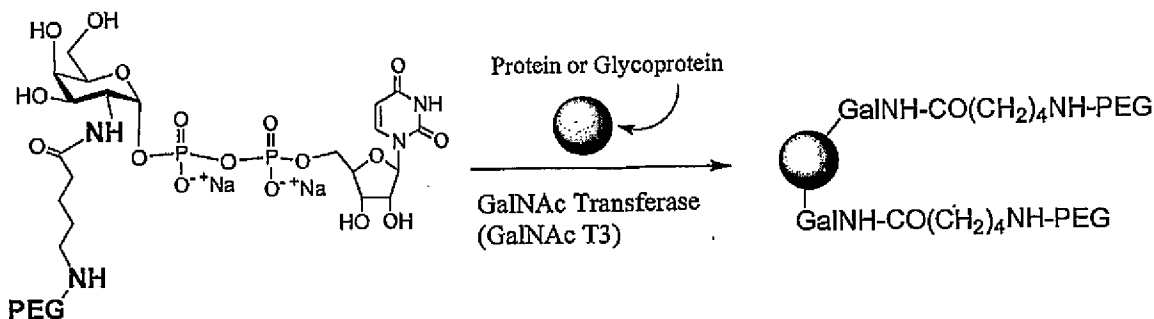
25   In each of the exemplary embodiments set forth above, one or more additional chemical or enzymatic modification steps can be utilized following the conjugation of the

modified sugar to the peptide. In an exemplary embodiment, an enzyme (*e.g.*, fucosyltransferase) is used to append a glycosyl unit (*e.g.*, fucose) onto the terminal modified sugar attached to the peptide. In another example, an enzymatic reaction is utilized to "cap" sites to which the modified sugar failed to conjugate. Alternatively, a chemical reaction is

5   utilized to alter the structure of the conjugated modified sugar. For example, the conjugated modified sugar is reacted with agents that stabilize or destabilize its linkage with the peptide component to which the modified sugar is attached. In another example, a component of the modified sugar is deprotected following its conjugation to the peptide. One of skill will appreciate that there is an array of enzymatic and chemical procedures that are useful in the

10   methods of the invention at a stage after the modified sugar is conjugated to the peptide. Further elaboration of the modified sugar-peptide conjugate is within the scope of the invention.

Peptide Targeting With Mannose-6-Phosphate

In an exemplary embodiment the peptide is derivatized with at least one mannose-6-

15   phosphate moiety. The mannose-6-phosphate moiety targets the peptide to a lysosome of a cell, and is useful, for example, to target therapeutic proteins to lysosomes for therapy of lysosomal storage diseases.

Lysosomal storage diseases are a group of over 40 disorders which are the result of defects in genes encoding enzymes that break down glycolipid or polysaccharide waste

20   products within the lysosomes of cells. The enzymatic products, *e.g.*, sugars and lipids, are then recycled into new products. Each of these disorders results from an inherited autosomal or X-linked recessive trait which affects the levels of enzymes in the lysosome. Generally, there is no biological or functional activity of the affected enzymes in the cells and tissues of affected individuals. Table 5 provides a list of representative storage diseases and the

25   enzymatic defect associated with the diseases. In such diseases the deficiency in enzyme function creates a progressive systemic deposition of lipid or carbohydrate substrate in lysosomes in cells in the body, eventually causing loss of organ function and death. The genetic etiology, clinical manifestations, molecular biology and possibility of the lysosomal storage diseases are detailed in Scriver *et al.*, eds., THE METABOLIC AND MOLECULAR BASIS

30   OF INHERITED DISEASE, 7.sup.th Ed., Vol. II, McGraw Hill, (1995).

Table 5. Lysosomal storage diseases and associated enzymatic defects

Disease	Enzymatic Defect
Pompe disease	acid α-glucosidase (acid maltase)
MPSI* (Hurler disease)	α-L-iduronidase
MPSII (Hunter disease)	iduronate sulfatase
MPSIII (Sanfilippo)	heparan N-sulfatase
MPS IV (Morquio A)	galactose-6-sulfatase
MPS IV (Morquio B)	acid β-galactosidase
MPS VII (Sly disease)	β-glucoronidase
I-cell disease	N-acetylglucosamine-1-phosphotransferase
Schindler disease	α-N-acetylgalactosaminidase (α-galactosidase B)
Wolman disease	acid lipase
Cholesterol ester storage disease	acid lipase
Farber disease	lysosomal acid ceramidase
Niemann-Pick disease	acid sphingomyelinase
Gaucher disease	glucocerebrosidase
Krabbe disease	galactosylceramidase
Fabry disease	α-galactosidase A
GM1 gangliosidosis	acid β-galactosidase
Galactosialidosis	β-galactosidase and neuraminidase
Tay-Sach's disease	hexosaminidase A
Magakaryotic leukodystrophy	arylsulphatase a
Sandhoff disease	hexosaminidase A and B

*MPS = mucopolysaccaridosis

De Duve first suggested that replacement of the missing lysosomal enzyme with exogenous biologically active enzyme might be a viable approach to treatment of lysosomal

5   storage diseases (De Duve, *Fed. Proc.* **23**: 1045 (1964). Since that time, various studies have suggested that enzyme replacement therapy may be beneficial for treating various lysosomal storage diseases. The best success has been shown with individuals with type I Gaucher disease, who have been treated with exogenous enzyme (β-glucocerebrosidase), prepared from placenta (Ceredase™) or, more recently, recombinantly (Cerezyme™). It has been

10   suggested that enzyme replacement may also be beneficial for treating Fabry's disease, as well as other lysosomal storage diseases. *See*, for example, Dawson *et al.*, *Ped. Res.* **7**(8): 684-690 (1973) (*in vitro*) and Mapes *et al.*, *Science* **169**: 987 (1970) (*in vivo*). Clinical trials of enzyme replacement therapy have been reported for Fabry patients using infusions of normal plasma (Mapes *et al.*, *Science* **169**: 987-989 (1970)), α-galactosidase A purified from

15   placenta (Brady *et al.*, *N. Eng. J. Med.* **279**: 1163 (1973)); or α-galactosidase A purified from spleen or plasma (Desnick *et al.*, *Proc. Natl. Acad. Sci., USA* **76**: 5326-5330 (1979)) and have demonstrated the biochemical effectiveness of direct enzyme replacement for Fabry disease.

These studies indicate the potential for eliminating, or significantly reducing, the pathological glycolipid storage by repeated enzyme replacement. For example, in one study (Desnick *et al., supra*), intravenous injection of purified enzyme resulted in a transient reduction in the plasma levels of the stored lipid substrate, globotriasylceramide.

5   Accordingly, there exists a need in the art for methods for providing sufficient quantities of biologically active lysosomal enzymes, such as human α-galactosidase A, to deficient cells. Recently, recombinant approaches have attempted to address these needs, *see*, *e.g.*, U.S. Pat. No. 5,658,567; 5,580,757; Bishop *et al., Proc. Natl. Acad. Sci., USA.* **83**: 4859-4863 (1986); Medin *et al., Proc. Natl. Acad. Sci., USA.* **93**: 7917-7922 (1996); Novo, F. J., *Gene Therapy.* **4**: 488-492 (1997); Ohshima *et al., Proc. Natl. Acad. Sci.,* USA. **94**: 2540-2544 (1997); and Sugimoto *et al., Human Gene Therapy* **6**: 905-915, (1995). Through the mannose-6-phosphate mediated targeting of therapeutic peptides to lysosomes, the present invention provides compositions and methods for delivering sufficient quantities of biologically active lysosomal peptides to deficient cells.

15   Thus, in an exemplary embodiment, the present invention provides a peptide according to Table 7 that is derivatized with mannose-6-phosphate (Figure 24 and Figure 25). The peptide may be recombinantly or chemically prepared. Moreover, the peptide can be the full, natural sequence, or it may be modified by, for example, truncation, extension, or it may include substitutions or deletions. Exemplary proteins that are remodeled using a method of the present invention include glucocerebrosidase, β-glucosidase, α-galactosidase A, acid-α-glucosidase (acid maltase). Representative modified peptides that are in clinical use include, but are not limited to, Ceredase™, Cerezyme™, and Fabryzyme™. A glycosyl group on modified and clinically relevant peptides may also be altered utilizing a method of the invention. The mannose-6-phosphate is attached to the peptide via a glycosyl linking group. In an exemplary embodiment, the glycosyl linking group is derived from sialic acid. Exemplary sialic acid-derived glycosyl linking groups are set forth in Table 3, in which one or more of the "R" moieties is mannose-6-phosphate or a spacer group having one or more mannose-6-phosphate moieties attached thereto. The modified sialic acid moiety is preferably the terminal residue of an oligosaccharide linked to the surface of the peptide (Figure 26)

In addition to the mannose-6-phosphate, the peptides of the invention may be further derivatized with a moiety such as a water-soluble polymer, a therapeutic moiety, or an additional targeting moiety. Methods for attaching these and other groups are set forth herein. In an exemplary embodiment, the group other than mannose-6-phosphate is attached

5   to the peptide via a derivatized sialic acid derivative according to Table 3, in which one or more of the "R" moieties is a group other than mannose-6-phosphate.

In an exemplary embodiment, a sialic acid moiety modified with a Cbz-protected glycine-based linker arm is prepared. The corresponding nucleotide sugar is prepared and the Cbz group is removed by catalytic hydrogenation. The resulting nucleotide sugar has an

10   available, reactive amine that is contacted with an activated mannose-6-phosphate derivative, providing a mannose-6-phosphate derivatized nucleotide sugar that is useful in practicing the methods of the invention.

As shown in the scheme below (scheme 15), an exemplary activated mannose-6-phosphate derivative is formed by converting a 2-bromo-benzyl-protected phosphotriester

15   into the corresponding triflate, *in situ*, and reacting the triflate with a linker having a reactive oxygen-containing moiety, forming an ether linkage between the sugar and the linker. The benzyl protecting groups are removed by catalytic hydrogenation, and the methyl ester of the linker is hydrolyzed, providing the corresponding carboxylic acid. The carboxylic acid is activated by any method known in the art. An exemplary activation procedure relies upon the

20   conversion of the carboxylic acid to the N-hydroxysuccinimide ester.

Scheme 15

CMP-SA-5-NHCOCH$_2$NH$_2$
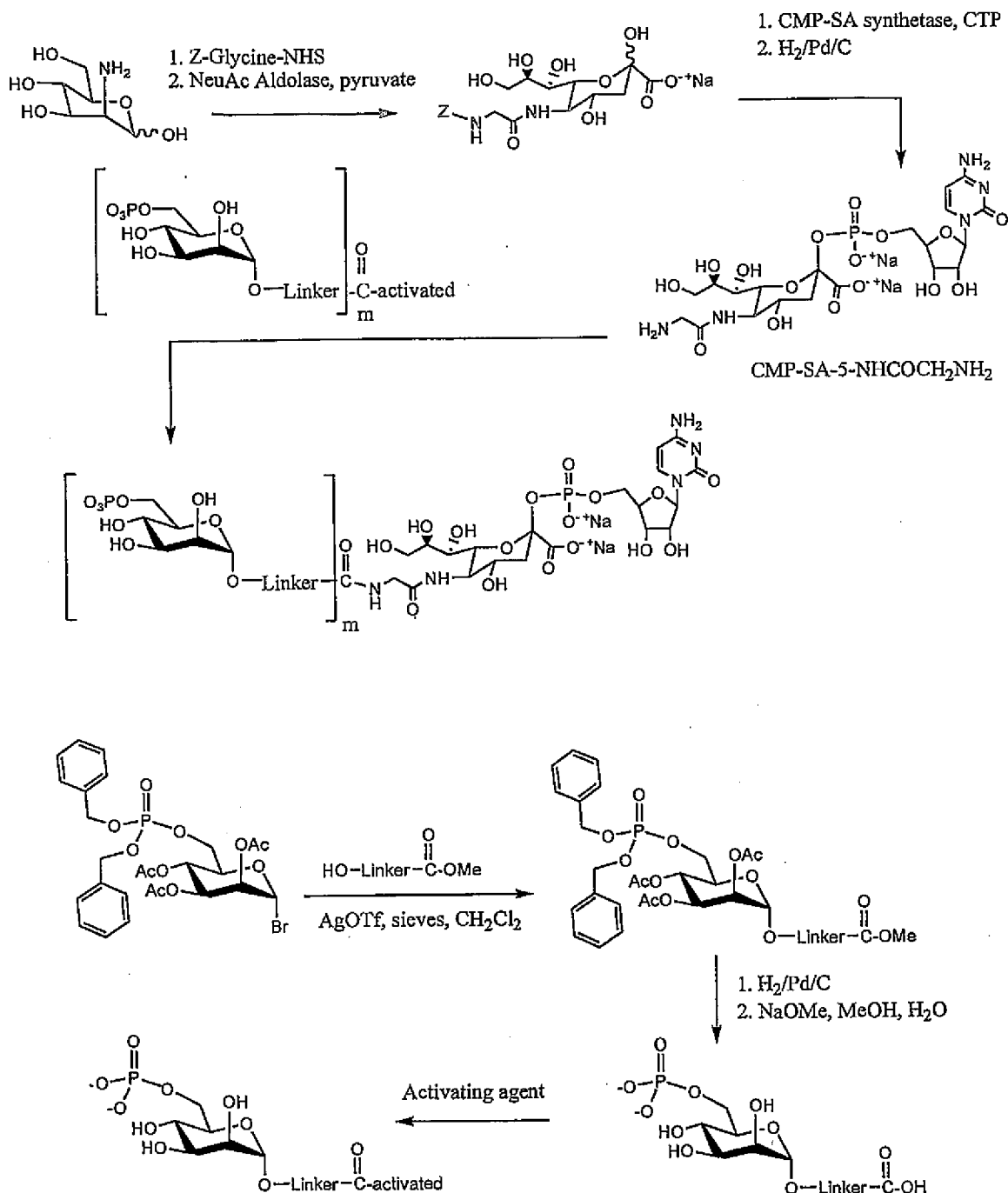
In another exemplary embodiment, as shown in the scheme below (scheme 16), a N-acetylated sialic acid is converted to an amine by manipulation of the pyruvyl moiety. Thus, the primary hydroxyl is converted to a sulfonate ester and reacted with sodium azide. The azide is catalytically reduced to the corresponding amine. The sugar is

subsequently converted to its nucleotide analogue and coupled, through the amine group, to
the linker arm-derivatized mannose-6-phosphate prepared as discussed above.

Scheme 16

CMP-SA-5-NHCOCH2NH2

5
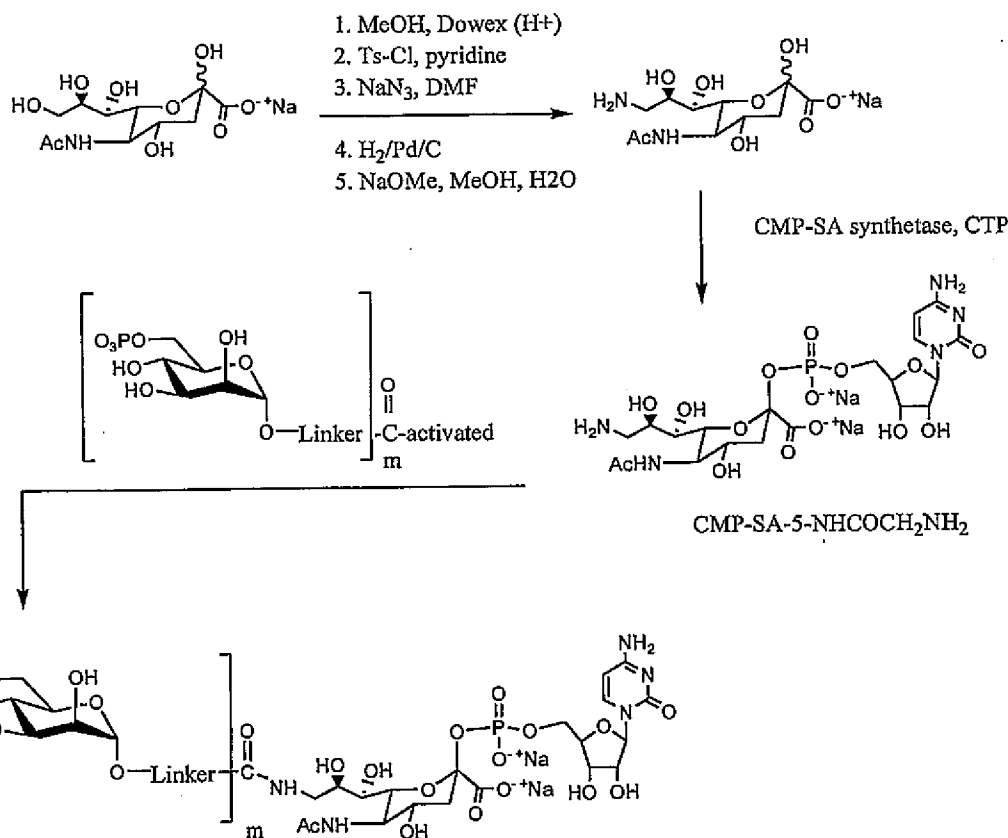
Peptides useful to treat lysosomal storage disease can be derivatized with other
targeting moieties including, but not limited to, transferrin (to deliver the peptide across the
blood-brain barrier, and to endosomes), carnitine (to deliver the peptide to muscle cells), and
phosphonates, e.g, bisphosphonate (to target the peptide to bone and other calciferous

10   tissues). The targeting moiety and therapeutic peptide are conjugated by any method
discussed herein or otherwise known in the art.

In an exemplary embodiment, the targeting agent and the therapeutic peptide are
coupled via a linker moiety. In this embodiment, at least one of the therapeutic peptide or the
targeting agent is coupled to the linker moiety via an intact glycosyl linking group according

15   to a method of the invention. In an exemplary embodiment, the linker moiety includes a
poly(ether) such as poly(ethylene glycol). In another exemplary embodiment, the linker

moiety includes at least one bond that is degraded *in vivo*, releasing the therapeutic peptide from the targeting agent, following delivery of the conjugate to the targeted tissue or region of the body.

In yet another exemplary embodiment, the *in vivo* distribution of the therapeutic
5   moiety is altered via altering a glycoform on the therapeutic moiety without conjugating the therapeutic peptide to a targeting moiety. For example, the therapeutic peptide can be shunted away from uptake by the reticuloendothelial system by capping a terminal galactose moiety of a glycosyl group with sialic acid (or a derivative thereof) (Figures 24 and 27). Sialylation to cover terminal Gal avoids uptake of the peptide by hepatic asialoglycoprotein
10   (ASGP) receptors, and may extend the half life of the peptide as compared with peptides having only complex glycan chains, in the absence of sialylation.

II. Peptide/Glycopeptides of the Invention

In one embodiment, the present invention provides a composition comprising multiple
15   copies of a single peptide having an elemental trimannosyl core as the primary glycan structure attached thereto. In preferred embodiments, the peptide may be a therapeutic molecule. The natural form of the peptide may comprise complex N-linked glycans or may be a high mannose glycan. The peptide may be a mammalian peptide, and is preferably a human peptide. In some embodiments the peptide is selected from the group consisting of an
20   immunoglobulin, erythropoietin, tissue-type activator peptide, and others (See Figure 28).

Exemplary peptides whose glycans can be remodeled using the methods of the invention are set forth in Figure 28.

Table 6. Preferred peptides for glycan remodeling

Hormones and Growth Factors	Receptors and Chimeric Receptors
G-CSF	CD4
GM-CSF	Tumor Necrosis Factor receptor (TNF-R)
TPO	TNF-R:IgG Fc fusion
EPO	Alpha-CD20
EPO variants	PSGL-1
FSH	Complement
HGH	GlyCAM or its chimera
insulin	N-CAM or its chimera
alpha-TNF	Monoclonal Antibodies (Immunoglobulins)
Leptin	MAb-anti-RSV
human chorionic gonadotropin	MAb-anti-IL-2 receptor
Enzymes and Inhibitors	MAb-anti-CEA
TPA	MAb-anti-glycoprotein IIb/IIIa
TPA variants	MAb-anti-EGF
Urokinase	MAb-anti-Her2
Factors VII, VIII, IX, X	MAb-CD20
DNase	MAb-alpha-CD3
Glucocerebrosidase	MAb-TNFα
Hirudin	MAb-CD4
α1 antitrypsin (α1 protease inhibitor)	MAb-PSGL-1
Antithrombin III	Mab-anti F protein of Respiratory Syncytial Virus
Acid α-glucosidase (acid maltase)	Anti-thrombin-III
α galactosidase A	Cells
α-L-iduronidase	Red blood cells
Urokinase	White blood cells (e.g., T cells, B cells,
Cytokines and Chimeric Cytokines	dendritic cells, macrophages, NK cells,
Interleukin-1 (IL-1), 1B, 2, 3, 4	neutrophils, monocytes and the like)
Interferon-alpha (IFN-alpha)	Stem cells
IFN-alpha-2b	Others
IFN-beta	Hepatits B surface antigen (HbsAg)
IFN-gamma	
IFN-omega	
Chimeric diphtheria toxin-IL-2	

Table 7. Most preferred peptides for glycan remodeling

Alpha-galactosidase A	Interleukin-2 (IL-2)
Alpha-L-iduronidase	Factor VIII
Anti-thrombin-III	hrDNase
Granulocyte colony stimulating factor (G-CSF)	Insulin
Interferon α	Hepatitis B surface protein (HbsAg)
Interferon β	Human Growth Hormone (HGH)
Interferon omega	Human chorionic gonadotropin
	Urokinase

Factor VII clotting factor	TNF receptor-IgG Fc fusion (Enbrel™)
Factor IX clotting factor	MAb-Her-2 (Herceptin™)
Follicle Stimulating Hormone (FSH)	MAb-F protein of Respiratory
Erythropoietin (EPO)	Syncytial Virus (Synagis™)
Granulocyte-macrophage colony	MAb-CD20 (Rituxan™)
stimulating factor (GM-CSF)	MAb-TNFα (Remicade™)
Interferon γ	MAb-Glycoprotein IIb/IIIa (Reopro™)
α₁ protease inhibitor (α₁ antitrypsin)	
Tissue-type plasminogen activator (TPA)	
Glucocerebrosidase (Cerezyme™)	

A more detailed list of peptides useful in the invention and their source is provided in Figure 28.

Other exemplary peptides that are modified by the methods of the invention include

5   members of the immunoglobulin family (*e.g.*, antibodies, MHC molecules, T cell receptors, and the like), intercellular receptors (*e.g.*, integrins, receptors for hormones or growth factors and the like) lectins, and cytokines (*e.g.*, interleukins). Additional examples include tissue-type plasminogen activator (TPA), renin, clotting factors such as Factor VIII and Factor IX, bombesin, thrombin, hematopoietic growth factor, colony stimulating factors, viral

10   antigens, complement peptides, α1-antitrypsin, erythropoietin, P-selectin glycopeptide ligand-1 (PSGL-1), granulocyte-macrophage colony stimulating factor, anti-thrombin III, interleukins, interferons, peptides A and C, fibrinogen, herceptin™, leptin, glycosidases, among many others. This list of peptides is exemplary and should not be considered to be exclusive. Rather, as is apparent from the disclosure provided herein, the methods of the

15   invention are applicable to any peptide in which a desired glycan structure can be fashioned.

The methods of the invention are also useful for modifying chimeric peptides, including, but not limited to, chimeric peptides that include a moiety derived from an immunoglobulin, such as IgG.

Peptides modified by the methods of the invention can be synthetic or wild-type

20   peptides or they can be mutated peptides, produced by methods known in the art, such as site-directed mutagenesis. Glycosylation of peptides is typically either N-linked or O-linked. An exemplary N-linkage is the attachment of the modified sugar to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for

enzymatic attachment of a carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a peptide creates a potential glycosylation site. As described elsewhere herein, O-linked glycosylation refers to the attachment of one sugar (e.g., N-acetylgalactosamine, galactose, mannose, GlcNAc, glucose, fucose or xylose)

5 to a hydroxy side chain of a hydroxyamino acid, preferably serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Several exemplary embodiments of the invention are discussed below. While several of these embodiments use peptides having names having trademarks, and other specific peptides as the exemplary peptide, these examples are not confined to any specific peptide.

10 The following exemplary embodiments are contemplated to include all peptide equivalents and variants of any peptide. Such variants include, but are not limited to, adding and deleting N-linked and O-linked glycosylation sites, and fusion proteins with added glycosylation sites. One of skill in the art will appreciate that the following embodiments and the basic methods disclosed therein can be applied to many peptides with equal success.

15 In one exemplary embodiment, the present invention provides methods for modifying Granulocyte Colony Stimulating Factor (G-CSF). Figures 29A to 29G set forth some examples of how this is accomplished using the methodology disclosed herein. In Figure 29B, a G-CSF peptide that is expressed in a mammalian cell system is trimmed back using a sialidase. The residues thus exposed are modified by the addition of a sialic acid-

20 poly(ethylene glycol) moiety (PEG moiety), using an appropriate donor therefor and ST3Gal1. Figure 29C sets forth an exemplary scheme for modifying a G-CSF peptide that is expressed in an insect cell. The peptide is modified by adding a galactose moiety using an appropriate donor thereof and a galactosyltransferase. The galactose residues are functionalized with PEG via a sialic acid-PEG derivative, through the action of ST3Gal1. In

25 Figure 29D, bacterially expressed G-CSF is contacted with an N-acetylgalactosamine donor and N-acetylgalactosamine transferase. The peptide is functionalized with PEG, using a PEGylated sialic acid donor and a sialyltransferase. In Figure 29E, mammalian cell expressed G-CSF is contacted with a sialic acid donor that is modified with levulinic acid, adding a reactive ketone to the sialic acid donor. After addition to a glycosyl residue on the

30 glycan on the peptide, the ketone is derivatized with a moiety such as a hydrazine- or amine-PEG. In Figure 29F, bacterially expressed G-CSF is remodeled by contacting the peptide

with an endo-GalNAc enzyme under conditions where it functions in a synthetic, rather than a hydrolytic manner, thereby adding a PEG-Gal-GalNAc molecule from an activated derivative thereof. Figure 29G provides another route for remodeling bacterially expressed G-CSF. The polypeptide is derivatized with a PEGylated N-acetylgalactosamine residue by

5   contacting the polypeptide with an N-acetylgalactosamine transferase and an appropriate donor of PEGylated N-acetylgalactosamine.

In another exemplary embodiment, the invention provides methods for modifying Interferon α-14C (IFNα14C), as shown in Figures 30A to 30N. The various forms of IFNα are disclosed elsewhere herein. In Figure 30B, IFNα14C expressed in mammalian cells is

10   first treated with sialidase to trim back the sialic acid units thereon, and then the molecule is PEGylated using ST3Gal3 and a PEGylated sialic acid donor. In Figure 30C, N-acetylglucosamine is first added to IFNα14C which has been expressed in insect or fungal cells, where the reaction is conducted via the action of GnT-I and/or II using an N-acetylglucosamine donor. The polypeptide is then PEGylated using a galactosyltransferase

15   and a donor of PEG-galactose. In Figure 30D, IFNα14C expressed in yeast is first treated with Endo-H to trim back the glycosyl units thereon. The molecules is galactosylated using a galactosyltransferase and a galactose donor, and it is then PEGylated using ST3Gal3 and a donor of PEG-sialic acid. In Figure 30F, IFNα14C produced by mammalian cells is modified to inched a PEG moiety using ST3Gal3 and a donor of PEG-sialic acid. In Figure 30G,

20   IFNα14C expressed in insect of fungal cells first has N-acetylglucosamine added using one or more of GnT-I, II, IV, and V, and an N-acetylglucosamine donor. The protein is subsequently galactosylated using an appropriate donor and a galactosyltransferase. Then, IFNα14C is PEGylated using ST3Gal3 and a donor of PEG-sialic acid. In Figure 30H, yeast produced IFNα14C is first treated with mannosidases to trim back the mannosyl groups. N-

25   acetylglucosamine is then added using a donor of N-acetylglucosamine and one or more of GnT-I, II, IV, and V. IFNα14C is further galactosylated using an appropriate donor and a galactosyltransferase. Then, the polypeptide is PEGylated using ST3Gal3 and a donor of PEG-sialic acid. In Figure 30I, NSO cell expressed IFNα14C is modified by capping appropriate terminal residues with a sialic acid donor that is modified with levulinic acid,

30   thereby adding a reactive ketone to the sialic acid donor. After addition to a glycosyl residue of the peptide, the ketone is derivatized with a moiety such as a hydrazine- or amine- PEG.